NEURAL REPAIR BY ENHANCING ENDOGENOUS HIPPOCAMPAL NEUROGENESIS FOLLOWING TRAUMATIC BRAIN INJURY

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Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Anatomy and Cell Biology,
Indiana University

October 2019

Accepted by the Graduate Faculty of Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ACKNOWLEDGEMENT

It has been a fantastic journey to pursue my Ph.D. degree at Indiana University School of Medicine. First of all, I would like to express my sincere gratitude to my mentor Dr. Jinhui Chen for his continuous guidance and generous support throughout my five years in the laboratory. He has shown great patience with me, taught me to ask and answer fundamental questions, witnessed my growth, and celebrated every single milestone I have achieved. I could not have asked for a better mentor than him.

Secondly, I would acknowledge my amazing research committee, Dr. Kathryn Jones, Dr. Jason Meyer, Dr. Karen Pollok, and Dr. Xiao-Ming Xu for their constant inspirations, kind encouragements, and crucial advice. They have helped to fine-tune my study and keep me on the right track. I have genuinely enjoyed the training with them and would benefit from it for a lifetime.

Thirdly, I truly appreciate the support from members of Chen Lab, especially Dr. Xiang Gao, Mr. Fang Yuan, and Ms. Shu Zhao. They have not only taught me with every single technique in the laboratory but also made the laboratory feels like a home away from home. It has been a terrific five-year to work in such a collaborative environment.

Additionally, I would like to thank the Indiana Spinal Cord and Brain Injury Research Group, Stark Neurosciences Research Institute, and Department of Anatomy and Cell Biology for providing the essential facilities and helpful discussions.

I would not have lived such a delightful life in Indianapolis without my dearest friends. Dr. Yu-Hsiang Chen, Dr. Emma Longworth-Mills, and Dr. Lakshmi Prabhu from my IBMG program 2013 cohort have been gorgeous friends whom we have shared ups and downs and explored the city a lot together. Dr. Chen Chen and Dr. Xingjie Ping have been loyal friends with whom we laughed and worried together, we shared our minds and emotions, we cheered up each other every day. My friends have not only lightened up my Ph.D. life and constantly inspired me, but also made me a better person.

Last but not least important, I would like to acknowledge my wonderful parents, Mr. Xueyong Wang and Mrs. Lanfeng Liu for their unconditional love and constant support throughout my life. I truly appreciate the trust and respect they gave me during my growing up years. They have set excellent examples for me. Their passionate attitudes to life and work inspired me. I would not have achieved any of my accomplishments without them.

Words are powerless to express my gratitude. I could not make a full list of people that have helped me in one way or the other. With the enormous help obtained, I have completed my Ph.D. study and am very much excited about the next chapter of my life to come.

Xiaoting Wang

NEURAL REPAIR BY ENHANCING ENDOGENOUS HIPPOCAMPAL NEUROGENESIS FOLLOWING TRAUMATIC BRAIN INJURY

Traumatic brain injury (TBI) is a critical public health issue in the United States, affecting about 2.8 million people annually. Extensive cell death and neural degeneration directly and diffusively caused by the initial mechanical insult results in a wide range of neurological complications post-trauma. Learning and memory dysfunction is one of the most common complains. Hippocampal neuronal loss, together with other mechanisms, largely contributes to learning and memory impairment as well as other cognitive dysfunctions post-trauma. To date, no FDA-approved drug is available to target cell death or improve learning and memory following TBI. It is of great interest to develop alternative approaches targeting neural repair instead.

Neural stem/progenitor cells (NSCs) in the adult hippocampus undergo life-long neurogenesis supporting learning and memory functions, thus hold great promise for post-traumatic neuronal replacement. The previous studies demonstrated that TBI transiently increase NSC proliferation. However, it is debated on whether TBI affects neurogenesis. The mechanism of TBI-enhanced NSC proliferation remains elusive. In the current studies, I have investigated post-traumatic neurogenesis after different injury severities, evaluated integration of post-injury born neurons, illustrated a molecular mechanism mediating TBI-enhanced NSC proliferation, proposed a *de novo* state of NSCs, and tested

effects of a pharmacological approach on spatial learning and memory function recovery.

My results demonstrated that post-traumatic neurogenesis is affected by injury severities, partially explained the pre-existing inconsistency among works from different groups. Post-injury born neurons integrate in neural network and receive local and distal inputs. TBI promotes functional recruitment of post-injury born neurons into neural circuits. Mechanistically, mechanistic target of rapamycin (mTOR) pathway is required primarily for TBI-enhanced NSC proliferation; NSCs feature a *de novo* alert state, in which NSCs are reversibly released from quiescence and primed for proliferation. Furthermore, my data demonstrated a beneficial role of ketamine in improving post-traumatic spatial learning possibly by activating mTOR signal in NSCs and/or promoting neuronal activity of post-injury born neurons. Together, my data support the feasibility of neurogenesis mediated neuronal replacement, provide a target for enhancing post-traumatic NSC proliferation and subsequent neurogenesis, and prove a potential pharmacological approach benefiting post-traumatic functional recovery in learning and memory.

Xiao-Ming Xu, Ph.D., Chair

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LIST OF ABBREVIATIONS

alNSC	Alert neural stem cells
aNSC	Active neural stem cells
ASLV	Avian sarcoma and leukosis virus
AT_2	Angiotensin receptor 2
AP	Anterior-posterior
BDNF	Brain-derived neurotrophic factor
BMP	Bone morphogenetic proteins
BrdU	5-bromo-2'-deoxyuridine
CCI	Controlled cortical impact
DAPI	4′,6-diamidino-2-phenylindole
Dcx	Doublecortin
DHF	7,8-dihydroxyflacvone
DREADD	Designed receptor exclusively activated by designed drugs
dSub	Dorsal subiculum
EC	Entorhinal cortex
EC II/V	Entorhinal cortex layer II/V
EnvA	Envelope protein A
ERK	Extracellular signal-activated kinase
EPO	Erythropoietin
FGF2	Fibroblast growth factor-2
FJB	Fluoro-Jade B
FPI	Fluid percussion impact
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HCl	Hydrochloride acid
HDG	Hippocampal dentate gyrus
IEG	Immediate early gene
IGF1	Insulin-like growth factor-1
IL-4/6	Interleukin-4/6
i.p.	Intraperitoneally
IPC	Intermediate progenitor cells
LM	Lateral-medial
MAPK	Mitogen-activated protein kinase
MHb	Medial habenular nuclei
ML	Molecular layer
MS	Medial septal nucleus
MSC	Mesenchymal stromal cell
MWM	Morris water maze
mTOR	Mechanistic target of rapamycin
NDB	Nucleus of the diagonal band of Broca
NG2	Neural/glial 2 proteoglycan
NPC	Neural progenitor cells
NSC	Neural stem cells

OB	Olfactory bulb
OPN	Osteopontin
PFA	Paraformaldehyde
PBS	Phosphate-buffered saline
PI3K/Akt	Phosphoinositide 3 kinase/Protein kinase B
PP	Perforant path
PRH	Perirhinal cortex
PTEN	Phosphatase and tensin homolog
qNSC	Quiescent neural stem cells
RGL	Radial glia-like cells
RSG	Retrosplenial cortex
SGZ	Subgranular zone
SVZ	Subventricular zone
SCI	Spinal cord injury
TBI	Traumatic brain injury
Tβ4	Thymosin β4
TGF β	Tumor growth factor β
TNF α	Tumor necrosis factor α
tPA	Tissue plasminogen activator
TrkB	Tropomyosin receptor kinase B
TVA	Tumor virus A
VEGF	Vascular endothelial growth factor
VSVG	Vesicular somatitis virus G glycoprotein

CHAPTER 1

INTRODUCTION

Background

As one of the most impactful socio-economic issues, traumatic brain injury (TBI) is rising in public awareness and attention on its high prevalence and serious sequelae. According to published data from the Centers for Disease Control and Prevention (CDC), about 2.5 million TBI-associated hospital visits are reported each year (Thomas R. Frieden, 2013). Death occurs in nearly 52,000 patients, while survivors are subjected to physical disabilities and a wide range of neurological complications including cognitive deficits, post-traumatic seizure, Alzheimer's disease, Parkinson's disease and various psychiatric diseases as well (Prigatano, 2005, Salmond and Sahakian, 2005, Vespa et al., 2010, Sivanandam and Thakur, 2012, Marras et al., 2014, Perry et al., 2015). Consequently, quality of life after TBI is largely compromised, especially for pediatric TBI cases (McCarthy et al., 2005, Hung et al., 2014). Although multiple pathological changes have been observed after TBI in both human subjects (by multiple neuroimaging techniques (Bodanapally et al., 2015)), and in animal models (by histological analysis (McIntosh et al., 1998)), effective therapy against TBI is an unmet clinical need.

Diffuse axonal injury, dendritic damage, and neuronal cell loss together directly cause neurodegeneration and disrupt intrinsic connectivity network in the brain (Sharp et al., 2014, Lu et al., 2005, Hall et al., 2008, Saatman et al., 2006),

accompanied by indirect impacts, such as neuroinflammation and neurovascular destruction (Nortje and Menon, 2004), which further deteriorates the brain injury. Cell death that affects a large number of areas in the brain is one of the most detrimental events following TBI (Saatman et al., 2006, Lu et al., 2005, Hall et al., 2008). Depending on the compromised region, neuronal loss can contribute to disconnection in neurocircuitries regulating body movement, cognitive and/or emotion functions. Thus, approaches aimed at neuroprotection or neural repair against cell death are urgently desired. Although numerous attempts and investigations have been conducted on neuroprotection reagent development (Alzheimer and Werner, 2002, Xiong et al., 2008, Xiong et al., 2007, Xiong et al., 2009, Wu et al., 2014, Chen et al., 2015), there have been no successes as of yet in the clinic. As an alternative approach, enhancement of neural repair following TBI is of great interest in the field.

Stem cells, a powerful tool for regenerative medicine, no doubt hold great promise in the area of neural repair. Several types of non-neural human stem cells, such as umbilical cord blood stem cells, mesenchymal stem cells, and marrow stromal stem cells, have been transplanted into rodent TBI models. Although there was improvement in functional outcomes, it was only related to the neuroprotective effects through structural and/or nutritional support rather than direct neural repair (Mastro-Martinez et al., 2015, Mahmood et al., 2005, Sykova and Jendelova, 2005). The discovery of neural stem/progenitor cells (NSC) in adult animals and humans provides ideal resources and hope for authentic network replenishment and restoration (Ming and Song, 2005). Both

transplantation of *in vitro* expanded NSCs and intervention on endogenous NSCs are considered as potential therapeutics. Several groups have demonstrated that NSC proliferation in the adult brain is promoted by TBI (Dash et al., 2001, Kernie et al., 2001, Ramaswamy et al., 2005, Rice et al., 2003, Rola et al., 2006, Sun et al., 2005, Sun et al., 2007, Yu et al., 2008, Gao et al., 2009a, Gao and Chen, 2013, Chirumamilla et al., 2002), indicating an innate repair mechanism and suggesting interventions on endogenous NSC-mediated neurogenesis as a promising approach for neuronal replacement. Indeed, enhanced neurogenesis has shown correlation with improved cognitive function recovery post-trauma (Lu et al., 2005, Lu et al., 2007, Kleindienst et al., 2005, Lu et al., 2003, Sun et al., 2009, Xiong et al., 2009, Xiong et al., 2008), thus further illustrating the feasibility of repairing neuronal loss and restoring damaged behavioral functions by enhancing endogenous neurogenesis. However, there is still a discrepancy on neurogenesis level alteration after TBI, inquiries into properties of post-injury born neurons, demand on mechanistic studies of TBI-mediated NSC activities, and the urgent need for therapeutics targeting neurogenesis and cognitive function improvement after TBI. This body of work would focus on these gaps in the knowledge base and provide new perspectives and insight into a number of TBI related issues: (1) depict a baseline of TBI reshaped neurogenesis, (2) demonstrate the anatomical and functional integration of post-injury born neurons, (3) illustrate a mechanism of mammalian target of rapamycin (mTOR) signaling mediated TBI-enhanced NSC proliferation, and (4) investigate a

pharmacological approach focused on endogenous neurogenesis and cognitive functional improvement.

Hippocampal cell death and cognitive function deficits following TBI

As a complicated disease process, TBI is composed of initial insult caused primary injury, which is followed by prolonged secondary injury. Thus, damage is not restricted to focal site, but diffusively and progressively impacts a much larger area of the brain parenchyma (McIntosh et al., 1998). To reproduce pathological alterations in humans, several animal TBI models were developed. Although many of them were developed to induce TBI in medium-sized animals, like cats and ferrets, they were subsequently remodeled to fit rodents later. Although each TBI animal model replicates some of the different pathophysiological events observed in human cases, neuronal loss is one of the common features shared by all the models studied to date (Morales et al., 2005). Following injury, diffusive cell death occurs in various cortical and subcortical regions, among which the hippocampus is noteworthily vulnerable.

In 1989, Cortez and colleagues observed rapid neuronal counts loss in the CA2-CA3 region of the ipsilateral hippocampus within 24 h of lateral fluid percussion (FPI) injury. The effects lasted for at least 4 weeks when less residual neurons were detected (Cortez et al., 1989). Later on, hilar neuron death was selectively identified, and consequent granule neuron hyperactivity was recorded in the same injury model (Lowenstein et al., 1992). In 1993, hippocampal neuron loss was first associated with cognitive deficits after TBI, where a positive

correlation was demonstrated between hilar neuron survival after FPI and performances in Morris Water Maze test (Hicks et al., 1993). The temporal and spatial profile of hippocampal neuronal loss was then characterized in great detail for FPI-induced trauma (Hicks et al., 1996, Sato et al., 2001).

Although different from diffusive injury caused by FPI model, the focal injury induced by controlled cortical impact (CCI) model displays hippocampal cell loss in a similar pattern. In 1995, McIntosh group customized a CCI model for study in mice and observed damaged neurons at 48 h after injury in hippocampal CA2-CA3, CA3, and dentate gyrus regions. These injuries corresponded to memory deficits (Smith et al., 1995). The research was further expanded on to a longer temporal profile with neuronal dystrophy and necrotic and/or apoptotic dentate granule neuron death detected up to 4 weeks post-trauma (Colicos et al., 1996, Chen et al., 2003, Lu et al., 2005, Hall et al., 2008, Colicos and Dash, 1996). My colleagues pinpointed predominant death of immature but not mature neurons in the dentate gyrus and proved strong evidence that a necrotic but not apoptotic cell death was the underlying mechanism of immature neuron death. My colleagues previously found that the necrotic immature neuron death occurs intensively and promptly within 24 h after moderate CCI injury (Gao et al., 2008, Zhou et al., 2012). Although detailed spatial-temporal and mechanistic characterizations are not exactly the same among the different studies published in this area, the fact that a significant neuronal loss happens in the hippocampus and the loss is correlated with cognitive functions deficits, especially learning and memory dysfunction, after TBI is consistent across all the studies. Additionally, it

is relatively clear that the therapeutic window for preventing cell death is narrow for therapy must be received within 24 h of injury.

So far, around 30 clinical trials have been conducted on neuroprotective reagents to minimize neuronal death. However, none of them has yet to succeed (Kabadi and Faden, 2014). Alternatively, regenerative medicine, specifically stem cell therapy, provides a new route to promote neural repair instead. Current efforts have been largely focused on stem cell transplantation, and some promising studies have shown improved motor and/or learning functions in rodents. However, a few concerns regarding cell source, dosage, and delivery have greatly restricted clinical translation (Gennai et al., 2015). It is possible that utilization of endogenous NSCs could achieve neural repair and restore the injured hippocampus and at the same time bypass the barriers mentioned above.

Neurogenesis in the adult brain

Conventionally, the adult brain was considered as a non-regenerative organ. Neurogenesis was traditionally deemed to only occur during embryogenesis and postnatal development. It was in the early 1960s when cell proliferation was first detected in the adult mouse brain by [H^3]-thymidine labeling (Smart, 1961). Then a series of investigations proved that new neurons were also detected in adult rat brain (Altman and Das, 1965, Altman and Das, 1966, Altman, 1969). The Nottebohm group was the first to publish a series of papers that proved the functional involvement of adult neurogenesis in songbirds' learning capacity (Nottebohm, 2002). Thereafter, the significance of adult neurogenesis was finally

recognized and accepted. Currently, NSCs are widely studied in adult canaries (Alvarez-Buylla et al., 1998), rodents (Reynolds and Weiss, 1992), primates (Kornack and Rakic, 1999), and humans (Kukekov et al., 1999, Roy et al., 2000). Investigators in the areas of NSC research agree that adult neurogenesis is mainly restricted to two neurogenic niches: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Figure 1.1). NSCs in the adult SVZ continuously generate precursors, which differentiate to neuroblasts, migrate via the rostral migratory stream, eventually arrive at the olfactory bulb, and become interneurons there (Doetsch et al., 1999, Ming and Song, 2005). NSCs in the adult SGZ also generate precursors, which commit mainly to neuronal differentiation and subsequently produce immature neurons. Newborn immature neurons migrate shortly from SGZ to granule cell layer, grow an extensive dendritic arborization, and become mature granule neurons, which eventually integrate into pre-existing neural circuits (Figure 1.2 A) (Zhao et al., 2006, van Praag et al., 2002, Ming and Song, 2005, Cameron and McKay, 2001).

Functions of adult neurogenesis

The function of adult neurogenesis in both regions has been extensively studied via stimulatory or inhibitory interventions. In the SVZ, inhibition studies have suppressed adult neurogenesis using anti-mitotic reagents, focal irradiation, and genetic modifications respectively, and consequently odor discrimination deficits, short-term and long-term olfactory memory dysfunctions have been discovered

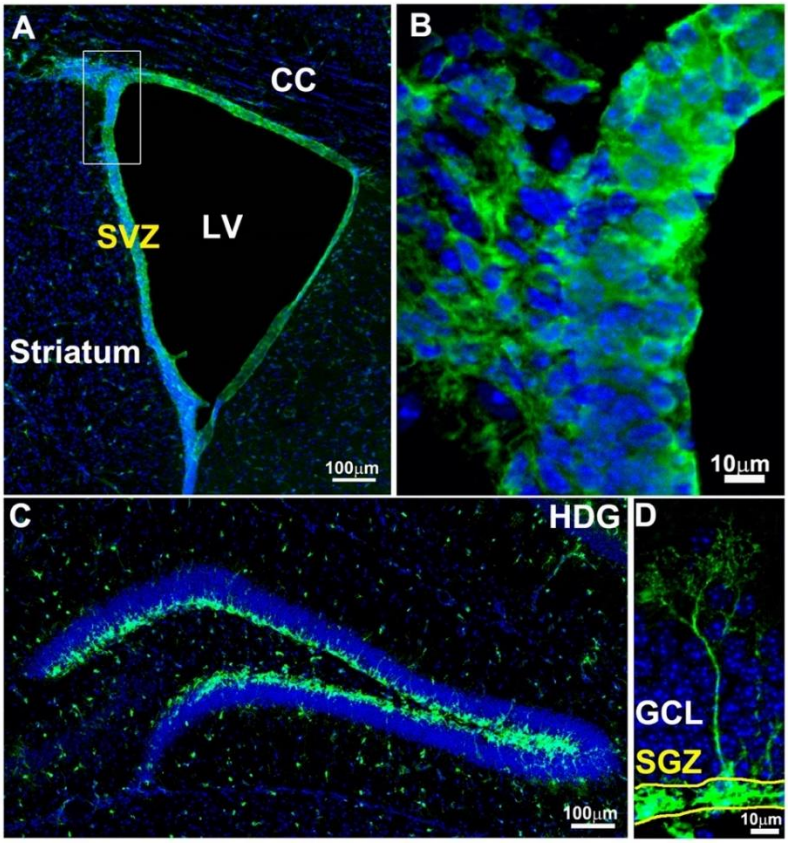


Figure 1.1: Neural stem cells (NSCs) in the adult mouse subventricular zone (SVZ) and subgranular zone (SGZ).

Coronal sections from adult Nestin-GFP transgenic mouse. (A) GFP staining (green) shows NSCs locate in the SVZ. (B) Enlarged image of A (indicated by white box) to show GFP positive NSCs in the SVZ in higher magnification. (C) GFP staining (green) shows NSCs locate in the SGZ. (D) Single cell image to show GFP positive NSCs in the SGZ in higher magnification. 4',6-diamidino-2-phenylindole (DAPI) staining of nuclei shows brain structure. CC: corpus callosum; GCL: granule cell layer; HDG: hippocampal dentate gyrus; LV: lateral ventricle; SGZ: subgranular zone; SVZ: subventricular zone. Adapted from Figure 29.1 of Chen and Wang et al. (Chen, 2018).

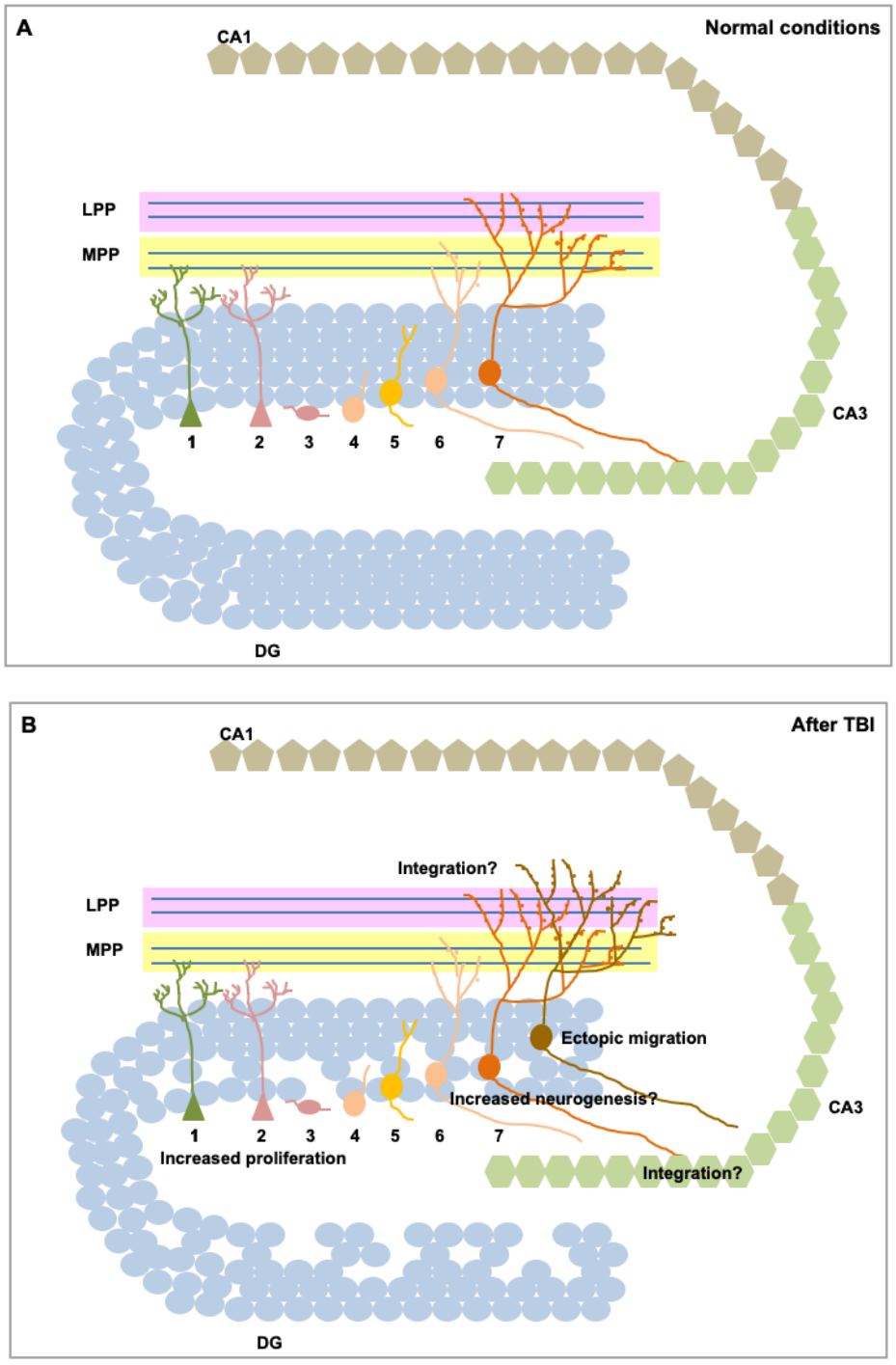


Figure 1.2: Adult NSCs and neurogenesis in the normal hippocampus and after TBI.

(A) Adult NSCs in the hippocampus support neurogenesis. 1: Radial-glial like NSC mainly remain quiescence in the SGZ, 2: Small proportion of RGL NSC constantly under activation to proliferate, 3: Active RGL NSCs generate neuronal lineage committed immediate progenitor cell, 4: immediate progenitor cell differentiate to neuroblast, 5: neuroblast become immature neuron in the SGZ, 6: immature neuron migrated from SGZ to inner GCL, extend dendrites towards ML and axons towards CA3, 7: after prolonged morphological and electrophysiological maturation, mature neurons integrate in pre-existing circuits. LPP: lateral perforant path; MPP: medial perforant path. (B) TBI alters neurogenesis at multiple steps, including increasing NSC proliferation and induce ectopic migration of post-injury born neurons. It is not agreed on if TBI increases neurogenesis, and it is not thoroughly studied if TBI alters post-injury born neuron integration.

correspondingly (Gheusi et al., 2000, Breton-Provencher et al., 2009, Arruda-Carvalho et al., 2014). In contrast, odor enrichment paradigm enhanced adult neurogenesis in the OB and promoted odor memory (Rochefort et al., 2002). Recent application of optogenetics further makes it possible to selectively excite adult-born OB neurons, and directly demonstrate their roles in facilitating learning and memory of odor discrimination in mice. Taken together, adult neurogenesis in the SVZ primarily participates in olfactory-associative functions.

In the SGZ, adult neurogenesis is considered essential for long-lasting learning and memory capacity, in line with the fundamental role of the hippocampus. Enriched environment similarly increased adult hippocampal neurogenesis and improved spatial memory performances correspondingly in Morris Water Maze test (Kempermann et al., 1997, Nilsson et al., 1999). An anti-mitotic reagent induced defective neurogenesis in the hippocampus resulted in trace memory formation deficits in a mouse model (Shors et al., 2001). Similarly, when adult hippocampus neurogenesis was ablated in focal irradiated mice or transgenic mice with neural precursor elimination, these mice displayed impairments in contextual fear conditioning behavior but with spatial memory remained intact (Saxe et al., 2006, Kitamura et al., 2009). Additionally, with compromised adult hippocampal neurogenesis, fear memory extended the period of hippocampus dependency before it was transferred to cortical structures for permanent storage (Kitamura et al., 2009). Likewise, it is suggested that adult hippocampal neurogenesis is required for memory clearance instead of formation, since memory retention was negatively correlated with adult

neurogenesis in mice (Feng et al., 2001, Akers et al., 2014). Together, adult hippocampal neurogenesis plays critical roles in learning and memory functions in the adulthood, presumably by facilitating the formation of new memory and clearance of old memory.

In an organism, adult neurogenesis is dynamically regulated by the physiological conditions and external stimuli. Aforesaid an enriched environment paradigm has been proven and is commonly used to increase neurogenesis in adult animals, similarly used methods include training with learning protocol and physical exercises such as running (Gould et al., 1999, van Praag et al., 1999, Kempermann et al., 1997, Nilsson et al., 1999, Rochefort et al., 2002). In contrast, age and stress were revealed to negatively modulate neurogenesis (Kuhn et al., 1996, Gould et al., 1997). These daily events together alter neurogenesis level dynamically, which coordinates with and determines animal behaviors. Likewise, under pathological conditions, the neurogenesis level is affected in a context-dependent manner. Brain injuries, such as stroke, epilepsy and TBI, have been reported to stimulate NSC proliferation and subsequently influence neurogenesis (Parent et al., 1997, Yagita et al., 2001, Parent et al., 2002, Yamashita et al., 2006, Kernie et al., 2001, Rice et al., 2003, Braun et al., 2002, Chirumamilla et al., 2002, Dash et al., 2001, Gao et al., 2009a). These observations suggest endogenous attempts of neuronal replacement mediated by NSCs. This innate repair machinery reveals a potential therapeutic target for clinical interferences and offers great promise for functional improvement.

TBI reshapes adult hippocampal neurogenesis

In the case of TBI, cell proliferation, including NSC proliferation, is widely observed to increase in rodent hippocampus (Dash et al., 2001, Chirumamilla et al., 2002, Braun et al., 2002, Ramaswamy et al., 2005, Rice et al., 2003, Rola et al., 2006, Sun et al., 2005, Kernie et al., 2001). A study from human hippocampal tissues validated the increase of NSC proliferation (Zheng et al., 2013). My colleagues further pinpointed that it is quiescent NSCs but not their progeny, the active (neural progenitor cells) NPCs, who were mainly activated by TBI in mice (Gao et al., 2009a). These observations demonstrated an innate repair attempt for neuronal replacement, mediated by NSCs in the damaged adult hippocampus. Nevertheless, NSC proliferation is just the starting point of neurogenesis. The increase of NSC proliferation does not necessarily guarantee an increase of neurogenesis or functional improvement. There is indeed a controversy in the published literature regarding how TBI affects the generation of mature granule neurons, in which increased, decreased, or unchanged neurogenesis have all been reported by individual groups (Braun et al., 2002, Rola et al., 2006, Sun et al., 2005, Sun et al., 2007, Gao and Chen, 2013, Bye et al., 2011, Dash et al., 2001, Kernie et al., 2001, Rice et al., 2003). To better understand the effects of TBI on neurogenesis, it is necessary to carefully evaluate not only the NSC proliferation but also the status of every step during neurogenesis. This is critical since the injury environment may also affect newborn neuron survival, migration, maturation, morphology development, as well as anatomical and functional integration (Figure 1.2 B).

Newborn neuron survival after TBI

Under physiological conditions, less than 25% adult-born granule neurons can survive the first three weeks after birth (Sierra et al., 2010, Christian et al., 2014, Tashiro et al., 2006). After trauma, how the injured environment impacts the survival rate of post-injury born neurons remains elusive. My colleagues previously reported rapid and dramatic cell death in the injured hippocampal dentate gyrus within 24 h after a moderate CCI injury. The cell death was revealed as selective loss of doublecortin-positive immature newborn neurons (Gao et al., 2008). The number of immature neurons returns to the level comparable to sham animals 2 weeks later, roughly the time it takes for the injury stimulated NSCs to undergo differentiation and turn to post-mitotic immature neurons (Wang et al., 2016a, Zhao et al., 2016). Although the increased NSC proliferation helps compensate for the cell loss, it is unknown whether the survival of post-injury born neurons is altered, which may also promote neuronal replacement. A few studies evaluated cell survival by labeling cell proliferation with 5-bromo-2'-deoxyuridine (BrdU) and then as a measure of survival, assessed the presence of BrdU-positive cells 2 weeks after TBI. Cell survival was unchanged in the SGZ (Rice et al., 2003, Bye et al., 2011). However, the identities of the cells that survived were not validated by specific cell type markers, thus the BrdU-positive cells that survived were not necessarily neurons. More investigations are needed to clarify if the survival rate of newborn neurons is altered by TBI.

Properties of post-injury born mature neurons

Once the cells survive, a short migration from the SGZ to inner granule cell layer (GCL) is also critical for newborn neurons to mature, properly integrate into local circuits, and establish synapses with appropriate axonal terminals. Recently, a study illustrated the aberrant localization of neurons newly born about 5 days post-injury, indicating ectopic migration in the injured environment (Villasana et al., 2015). My colleagues also revealed the migration issue of newborn neurons in moderate and severe CCI. They have observed that a large proportion of newborn neurons were misplaced in outer GCL or even mismigrated further to the border between GCL and molecular layer. These neurons stay misplaced after they fully mature (Wang et al., 2016a, Ibrahim et al., 2016). Adult-born granule neurons generally locate in the inner GCL. Thus the ectopic migration of newborn neurons may cause circuit disorganization and potentially contribute to post-traumatic epilepsy (Hunt et al., 2013).

Besides the aberrant migration issue, post-injury born neurons were also subjected to morphological alterations. Neurons born after CCI extended more proximal and less distal branches from their somas, and their dendritic arborizations showed more widespread morphologies. This morphology indicates a shift from synapses on distal inputs to synapses on proximal inputs, thus potentially disturbing original information flow. Whereas, the post-injury born neurons still maintained comparable electrophysiological properties compared to newborn neurons in sham animals (Villasana et al., 2015). The post-injury born neurons are able to fire action potentials responding to perforant path stimulation

and uptake retrograde tracer from CA3 pyramidal neurons. These observations demonstrated that post-injury born neurons extended their dendrites and formed synapses on perforant path and spread their axons to and formed synapses on CA3 pyramidal neurons (Sun et al., 2007, Villasana et al., 2015, Emery et al., 2005). Together, both similarities and differences were detected in neurons born after injury compared with newborn neurons in sham animals. However, more questions remain unanswered. Do the post-injury born neurons receive input signals from other brain regions besides perforant path like their counterparts in uninjured animals? Do they change the ratio of innervation from different regions? If so, will this alteration affect behavior outcomes? Do the post-injury born neurons exert neuronal activities? If so, how are they contributing to post-traumatic functional recovery? Further investigations are urgently needed to thoroughly elucidate the integration of post-injury born neurons and illustrate the functional relevance of post-traumatic neurogenesis to behavioral performances.

Post-traumatic neurogenesis level

Morphological and electrophysiological studies on individual post-injury born neurons elucidated some of their functional properties, however, it is still controversial of how TBI affects the production of mature neurons on a population level (Braun et al., 2002, Rola et al., 2006, Sun et al., 2005, Sun et al., 2007, Gao and Chen, 2013, Bye et al., 2011, Rice et al., 2003, Dash et al., 2001, Kernie et al., 2001). A few variables were identified in the contradictory studies after a thorough review of the specific approaches used by individual groups to

evaluate post-traumatic neurogenesis. These variations included injury model, time point post-injury, and cell fate tracing method, which collectively might together contribute to the existing discrepancy (Table 1). The first studies on post-traumatic neurogenesis were published in 2001 independently by two groups. Kernie and colleagues evaluated post-traumatic neurogenesis by a CCI model in adult mice. They traced cell proliferation by BrdU in the first week post injury, followed by the fate identification of the BrdU positive cells (i.e. cells that proliferated) at 60 days after injury. Their results demonstrated a 5-fold increase of BrdU-labeled cell proliferation in the injured hippocampus, and revealed co-labeling of BrdU with calbindin, proving mature neuron generation post-trauma (Kernie et al., 2001). In the meantime, Dash and colleagues reported similar results in adult rats. They induced TBI by CCI model and labeled proliferation for 9 days after injury. In their study, BrdU-positive cells were assessed acutely at 1 day after the last BrdU injection, which similarly exhibited a dramatic increase in dentate gyrus. Identities of BrdU-positive cells were determined at 3 weeks later by calbindin expression, and BrdU/calbindin double-labeled cells were also detected (Dash et al., 2001). Kernie and Dash demonstrated similar phenomena, including cell proliferation and neurogenesis induced by CCI. Whereas, the number of BrdU and calbindin double-labeled cells was not quantified or compared between injured and sham animals, so the question of how TBI remodels neurogenesis was not answered. In 2002, Braun and colleagues studied neurogenic cells by βIII-tubulin expression in adult rats after a weight drop injury. They observed an increase of the neurogenic cells at 3 and 7 days

Table 1: Summary of major studies on post-traumatic neurogenesis.

Post-traumatic Neurogenesis Level	Injury Model	Experiment Parameters	Species	Cell Label Strategy	Tissue Collection Time	Neurogenesis Markers	References
No quantification	CCI	4.4m/s, 1.0mm deformation	mouse	BrdU labeled for 1 week after injury	60 days post-injury	BrdU/Calbindin	Kernie et al., 2001
		6m/s, 2.75mm deformation	rat	BrdU labeled for 9 days after injury	30 days post-injury	BrdU/Calbindin	Dash et al., 2001
	weight drop	20g, 9cm		not labeled	7 days post-injury	βIII tubulin	Braun et al., 2002
Increase	FPI	2.1 ± 0.08 atm	rat	BrdU labeled at 2 days after injury	4 weeks post-injury	BrdU/NeuN	Sun et al., 2005
		2.2 ± 0.02 atm		BrdU labeled from 2 days to 5 days after injury	10 weeks post-injury	BrdU/FluoroGold	Sun et al., 2007
	FPI	2.16 ± 0.06 atm	rat	BrdU labeled at 18h and 20h after injury	15 days post-injury	BrdU/βIII tubulin	Rice et al., 2003
Unchanged	CCI	3.0m/s, 1.0mm deformation	mouse	BrdU labeled for 1 week after injury	5 weeks post-injury	BrdU/NeuN	Gao et al., 2013
	weight drop	450g, 2m, 3.6m/s	rat	BrdU labeled from 24h to 4days after injury	8 weeks post-injury	BrdU/NeuN	Bye et al., 2013
Decreased	CCI	4.5m/s, 1mm deformation	mouse	BrdU labeled for 1 week after injury	4 weeks post-injury	BrdU/NeuN	Rola et al., 2006

Adapted from Table 29.1 of Chen and Wang et al. (Chen, 2018).

post-trauma in the dentate gyrus (Braun et al., 2002). Collectively, these early investigations set the foundation for TBI-enhanced NSC proliferation. Although the proliferated NSCs can produce mature neurons after injury, how TBI changes the level of neurogenesis still remained elusive. To address this question, several other groups have followed up this work. Sun and colleagues reported increased neurogenesis in adult rats after FPI injury. They demonstrated increased neurogenesis by BrdU injection at 2 days after trauma and by assessing cell fate via neuronal nuclei (NeuN) double-labeling at 4 weeks after TBI (Sun et al., 2005). Their follow-up work further validated the increased neurogenesis at a long-term time point, 10 weeks after TBI (Sun et al., 2007). On the contrary, in a CCI injury study in adult mice, Rola *et al* reported decreased neurogenesis by BrdU labeling in the first week and fate tracing via BrdU and NeuN co-staining at 4 weeks post-trauma (Rola et al., 2006). Whereas using a similar paradigm, my colleagues instead detected unchanged neurogenesis following trauma by BrdU labeling in the first week, and cell fate determination with BrdU and NeuN co-labeling at 5 weeks after a moderate CCI in adult mice (Gao and Chen, 2013). Bye *et al* demonstrated in an adult rat traumatic axonal injury (TAI) model similar unchanged neurogenesis by BrdU labeling for the first 5 days and BrdU NeuN double positive cell quantification at 8 weeks post-trauma (Bye et al., 2011). Moreover, Rice *et al* studied neurogenesis by BrdU injection acutely at 18 h and 20 h after FPI induced TBI in adult rats, and co-labeling of BrdU with βIII-tubulin at 15 days later. Similarly, they did not observe any apparent difference in the injured SGZ compared to sham cohorts, while increased neurogenesis was

observed in the SVZ (Rice et al., 2003). Collectively, key factors that varied among different studies consisted of animal species, injury models, injury parameters, labeling strategies, and assessment time points (Table 1). Each of the individual variants by itself or the combination of any might contribute to the existed discrepancy in proving TBI-remodeled neurogenesis. The first part of chapter 2 in this body of work helped settle the controversy by pinpointing injury severity as a factor that can determine neurogenesis level following trauma. I illustrated that although post-traumatic NSC proliferation increases as injury severity elevates, only severe but not mild or moderate CCI increases neurogenesis in adult mice (Wang et al., 2016a). Further investigations are in need to determine how the other factors influence post-traumatic neurogenesis and to help elucidate a more detailed profile of how TBI reshapes neurogenesis.

Function of post-traumatic neurogenesis

Learning and memory dysfunction is one of the most common complaints by TBI patients. This is largely resulted from neurocircuitry disconnection in the hippocampus caused by neuronal loss and degeneration. Meanwhile, spontaneous recovery was observed in both humans and rodents, although by different degrees, indicating activation of the intrinsic neuroplasticity post-trauma (Schmidt et al., 1999, Prigatano, 1987). Post-traumatic neurogenesis represents one of the neuroplasticity mechanisms by taking the place of dead neurons and reconnecting the damaged circuits. In animal models, increase of post-traumatic neurogenesis by neurotrophic factors was positively correlated with cognitive

function improvement (Kleindienst et al., 2005, Lu et al., 2005, Lu et al., 2003, Wu et al., 2008, Lu et al., 2007, Sun et al., 2009). By loss-of-function method, other studies proved the participation of post-traumatic neurogenesis in the functional improvement. Approaches used include genetic ablation of neurogenic cells and systemic inhibition of cell proliferation, by which diminishment of spontaneous recovery was observed (Blaiss et al., 2011, Sun et al., 2015).

Additionally, investigations on individual post-injury born neurons have suggested their integration into the neural network, supporting the hypothesis that post-traumatic neurons can exert neuronal function and post-traumatic neurogenesis is beneficial for functional improvements (Sun et al., 2007, Emery et al., 2005, Villasana et al., 2015). However, although integration of post-injury born neurons was observed, more information is needed to determine the exact identities of pre- and post-synaptic neurons the post-injury born neurons connect with. As mentioned in the prior session, the ectopic migration and abnormal dendritic arborization were observed in post-injury born neurons, and aberrant neurogenesis is also considered a contributor of post-traumatic epilepsy (Villasana et al., 2015). It is essential to investigate if the post-injury born neurons are synapsing on proper targets and rebuilding a functional network that follows the intrinsic pattern or instead, misrepair the circuits and further disrupt the damaged organizations. With the recent invention of monosynaptic pseudorabies virus tracing system, the pre-synaptic inputs onto post-injury born neurons can be investigated, and thus help assess if the post-injury born neurons rewire the damaged circuits and/or disrupt the pre-existing network. In the second part of

chapter 2, I used this method and exhibited that post-injury born neurons are innervated by a variety of neurons, both locally within the hippocampus and distally from cortical and subcortical regions. More quantitative studies would help to understand if there is any alteration regarding the innervation pattern.

Moreover, the approaches used to study the function of post-traumatic neurogenesis have been either correlative analysis or ablation a population of cells, but not direct and selective demonstration of a cause-and-effect relationship between post-traumatic neurogenesis and functional outcomes. So far, there is a lack of direct evidence showing the functional recruitment of post-injury born neurons. No studies have displayed if specific inhibition of post-injury born neuron activity can block post-traumatic functional recovery or not. Thus the beneficial and/or maladaptive effects of post-traumatic neurogenesis need to be further validated. Recent development of the DREADD (designed receptors exclusively activated by designed drugs) and optogenetic systems are powerful tools for selective investigations on newly born neurons (Lee et al., 2014, Boyden, 2015). By assessing behavioral performances with exclusive excitation or silence of post-injury born neurons, it is possible to directly evaluate the beneficial and/or maladaptive functions of post-traumatic neurogenesis. Only when comprehensive evaluation on the functions of post-injury neurogenesis is achieved, can translational studies on endogenous NSCs and post-traumatic neurogenesis realistically be worth pursuing. This is a critical prerequisite for any potential clinical applications of and/or interventions on post-traumatic endogenous neurogenesis as a therapeutic treatment for TBI.

Mechanisms of TBI-induced neurogenesis

In normal conditions, NSC activity and neurogenesis is regulated by extracellular signals in the NSC niche of the hippocampal SGZ. Various cell types, blood vessels and extracellular matrix in the niche together regulate NSC activity through releasing neurotransmitters, secreting growth/neurotrophic factors and cytokines, providing direct cell-cell contact, and/or offering systemic factors (Figure 1.3 A). For instance, parvalbumin interneurons are critical for NSCs to maintain quiescent by tonic releasing GABA (Song et al., 2012). NPCs express delta-like 1 to activate Notch signal in NSCs and maintain NSC quiescence (Kawaguchi et al., 2013). Astrocytes can secrete IL-1 β and IL-6 to promote neuronal differentiation (Barkho et al., 2006). Microglia are known to shape neurogenesis by cleaning non-survived newborn neurons via phagocytosis (Sierra et al., 2010). The expression and secretion of various growth/neurotrophic factors and cytokines locally from cells within the niche or distally from the system and released from blood vessels plays critical roles in NSC quiescence maintenance, proliferation, neuronal differentiation, survival, and morphological development (Faigle and Song, 2013). After TBI, there are dramatic alterations in the cellular components, like immature neuron death, astrocyte reactivation, and microglia reactivation, which can alter signal profiles in the niche (Figure 1.3 B). It is still unclear which ones are primarily responsible for injury-enhanced NSC proliferation and differentiation.

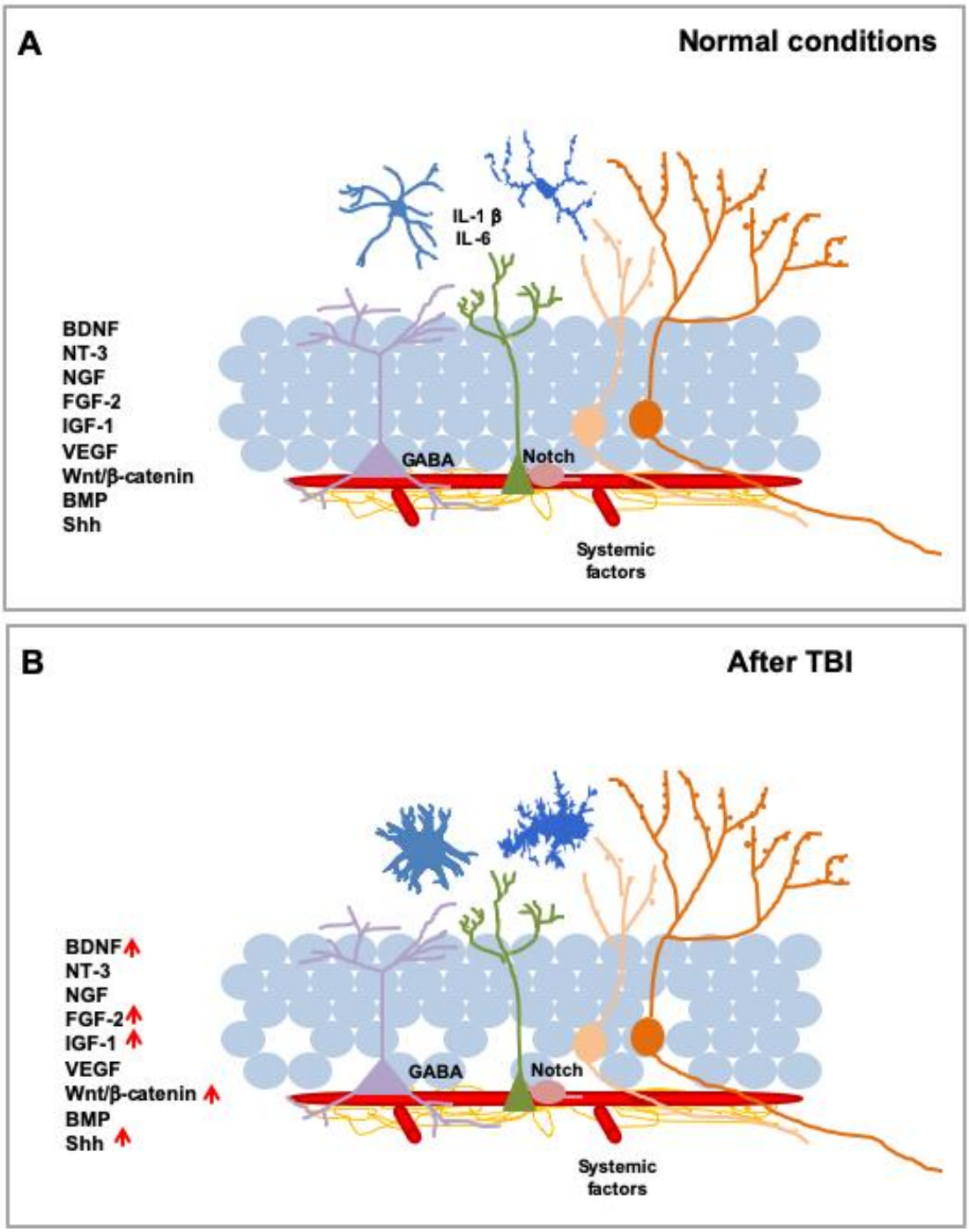


Figure 1.3: NSC niche in the normal hippocampus and after TBI.

(A) NSC niche in the normal hippocampus consist of their progenies, the immediate progenitor cells, immature and mature granule cells. Parvalbumin interneurons (light purple), astrocytes (grey-blue), and microglia (blue) around

NSCs (green) participate in NSC activity and neurogenesis regulation. Blood vessels (red) and extracellular matrix (yellow) provide systemic factors and other extracellular signals, respectively. Growth/neurotrophic factors and cytokines provided by multiple cell sources modulate neurogenesis. Modified from Figure 2 (Bond et al., 2015). (B) After TBI, a series of alterations in the NSC niche were reported, included but not limited to granule neuron death, astrocyte and microglia reactivation, and changes of a few growth/neurotrophic factors and cytokines.

Extracellular signals in NSC niche after TBI

All four classical pathways in stem cell biology have been reported to be involved in post-traumatic NSC activities and neurogenesis. Notch pathway, a main contributor to adult NSC maintenance and expansion (Ables et al., 2010, Ehm et al., 2010), was studied after TBI. Inhibitor of Notch1 pathway signaling, γ-secretase inhibitor, has been introduced to TBI animals and decreased NSC proliferation after injury (Kishimoto et al., 2012). On the contrary, Notch1 downstream target Hes1 has been reported to negatively regulate NSC proliferation and neurogenesis after TBI (Zhang et al., 2014). The role of Notch pathway in TBI-induced neurogenesis still needs to be clarified. The implications on other pathways are relatively limited. Wnt/β-catenin pathway normally enhances NSC proliferation and neuronal differentiation (Lie et al., 2005). After TBI, increased expression of Wnt/β-catenin pathway downstream target, survivin, has been observed in the hippocampus, which indicated the potential involvement of Wnt//β-catenin pathway in TBI-induced NSC proliferation (Zhang et al., 2013a). However, change of β-catenin itself or how Wnt/β-catenin signaling influences NSC proliferation after TBI has not been studied yet. Moreover, Sonic hedgehog (Shh) is another pathway normally regulating NSC proliferation and changed after TBI (Lai et al., 2003). Shh-responsive cells in the SVZ after CCI were increased in the ipsilateral brain, consistent with its role in enhancing NSC proliferation under normal conditions (Ahn and Joyner, 2005), while the correlation with NSC proliferation has not been determined (Mierzwa et al., 2014). Additionally, bone morphogenetic proteins (BMPs) are known to

promote gliogenesis and suppress neuronal fate specification, their mRNA expression was increased in SVZ and SGZ after TBI, but no functional relevance to neurogenesis after TBI has been evaluated (Logan et al., 2013, Lim et al., 2000). There are still a series of studies that need to be done to determine the dominant contributor to TBI-induced NSC proliferation or to unravel the combinational roles of each pathway.

Besides the extrinsic signals, neurotrophic factors and growth factors are another group of regulators of neurogenesis after TBI. They normally come from multiple cell sources and act on multiple steps of adult neurogenesis. A member of the neurotrophic factor family, brain-derived neurotrophic factor (BDNF), has been widely studied in the development of the nervous system, and is the most important factor involved in adult neurogenesis. Ablation of either BDNF or its receptor in hippocampal NSCs impaired NSC proliferation and reduced granule cell layer volume (Li et al., 2008). Meanwhile, BDNF plays an important role in promoting survival of the immature and mature neurons, and it is required for dendritic arborization development of new granule cells (Ghosh et al., 1994, Kirschenbaum and Goldman, 1995, Gao and Chen, 2009, Gao et al., 2009b). After TBI, both protein and mRNA levels of BDNF increase in the cortex and hippocampus (Grundy et al., 2000, Yang et al., 1996, Griesbach et al., 2004), indicating the involvement of BDNF in TBI pathophysiology. Previous study from my colleagues confirmed the administration of a BDNF receptor agonist successfully increased immature neuron survival post trauma, suggesting that BDNF plays a positive role in neuroplasticity after injury (Zhao et al., 2015b).

Beneficial effects of delayed exercises on increasing neurogenesis and functional recovery post trauma also correlated with BDNF up-regulation in the hippocampus (Griesbach et al., 2004).

Other than neurotrophic factors, growth factors, i.e. insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), are also crucial for NSC regulation and neurogenesis. IGF-1 positively regulates NSC proliferation and neurogenesis *in vitro* and *in vivo* in the adult hippocampus (Aberg et al., 2000, Arsenijevic and Weiss, 1998). IGF-1 and its receptor have exhibited increased expression in TBI animals in both the cortex and hippocampus (Madathil et al., 2010, Schober et al., 2010). Conditional overexpression of IGF-1 can enhance neurogenesis in the hippocampus after TBI and rescue dendritic impairments as well (Carlson et al., 2014). VEGF, secreted by astrocytes, microglia, neurons, and NSCs, is a potent angiogenic and neurogenic factor (Mosher et al., 2012). VEGF treatment can enhance NSC proliferation and neurogenesis in the adult hippocampus and improve hippocampus-dependent contextual fear conditioning (Segi-Nishida et al., 2008, Licht et al., 2011, Kirby et al., 2015). Post injury, VEGF expression is increased in the cortex and hippocampus. VEGF antisense oligonucleotide treatment impaired injury-induced neurogenesis, while administration of exogenous VEGF after TBI promoted neurogenesis in the DG and SVZ (Skold et al., 2005, Lu et al., 2011, Lee and Agoston, 2010, Thau-Zuchman et al., 2010). FGF-2 functions as another important regulator of adult neurogenesis (Woodbury and Ikezu, 2014). FGF-2 infusion increased cell proliferation and neurogenesis in the SVZ

and adult hippocampus; inducible FGF-2 receptor knockout in the adulthood, on the contrary, reduced neurogenesis in the hippocampus and compromised short-term memory (Kuhn et al., 1997, Stevens et al., 2012). After TBI, FGF-2 is also required for post-traumatic neurogenesis. FGF-2 deficient animals displayed decreased hippocampal neurogenesis and dentate gyrus volume post-trauma. Oppositely, FGF-2 overexpression and infusion increased hippocampal neurogenesis after TBI, respectively, which correlated to improved cognitive function recovery (Yoshimura et al., 2003, Sun et al., 2009). In Summary, BDNF, IGF-1, VEGF, and FGF-2, as neurogenic factors, all positively regulate neurogenesis in the normal and also injury environments. Investigations on direct administration of the above factors in TBI animals for functional recovery is strong evidence that their receptors are good targets for the development of small molecule analogs to achieve similar beneficial effects. However, interventions on any of these receptors has yet to succeed in clinical trials.

Intracellular signals in NSCs after TBI

With all the extracellular factors regulating NSC activity, it is extremely complicated to coordinate their alterations after TBI. The combination of their effects on post-traumatic neurogenesis and/or the dominant contributors to post-traumatic neurogenesis largely remain elusive. Therefore, the development of an optimal approach maximizing therapeutic effects is impeded. Thus, it might be more feasible to study the induction of an intracellular signaling pathway instead, since most of the neurotrophic and growth factors share the same pathways,

such as PI3K/Akt (Phosphoinositide 3 kinase/Protein kinase B), MAPK/ERK (Mitogen-activated protein kinase/Extracellular signal-activated kinase) for signal transduction (Huang and Reichardt, 2003, Dailey et al., 2005, Kowanetz and Ferrara, 2006, Chitnis et al., 2008). These involved signaling pathways, mainly responsible for cell survival and proliferation, are possible shortcuts to therapeutic augmentation of endogenous neurogenesis. Alterations of the PI3K/Akt pathway after TBI in the adult brain have been reported, as well as its involvement in treatment enhanced neuronal survival, neurogenesis, histological and behavioral outcome improvements (Chen et al., 2012, Wu et al., 2008, Wu et al., 2014). In terms of neurogenesis, administration of a cholesterol-lowering medication, simvastatin, exhibited a correlation between the up-regulation in PI3K/Akt pathway and increased neurogenesis (Wu et al., 2008). However, more investigation is needed to distinguish if there is a cause-and-effect relationship or is these are simply independent events. MAPK/ERK pathway activation has also been implicated in reducing apoptosis and increasing neurogenesis when activated after injury (Ma et al., 2011, Lu et al., 2011).

Since PI3K/Akt and MAPK/ERK converge on the mTOR (mammalian target of rapamycin, or mechanistic target of rapamycin) pathway downstream, it is of great interest to study mTOR signaling after TBI (Mendoza et al., 2011). Generally, mTOR pathway is a cellular hub controlling multiple events, including cell metabolism, growth, proliferation, survival, migration, etc. The mTOR pathway consists of the mTOR complex 1 (mTORC1), involved in most of the mTOR activities, and mTOR complex 2 (mTORC2), mainly controlling cell

survival and cytoskeleton. It responds to diverse extracellular signals, like nutrients, amino acids, growth factors, cytokines, and energy. Intracellularly, mTOR senses cellular energy status, oxidative states, and DNA damage (Meng et al., 2018). Its activation depends on PI3K/Akt signaling, and it is negatively regulated by PTEN (Figure 1.4 A). Its roles have been extensively studied in various diseases, such as cancers, diabetes, and neurodegenerative diseases (Laplante and Sabatini, 2012). Recently, mTOR has also gained attention in stem cell studies and neuroregeneration fields (Meng et al., 2018, Park et al., 2008, Liu et al., 2010, Liu et al., 2017).

mTOR signaling in TBI

After TBI, increased mTOR activation has been detected in the cortex and hippocampus in a mouse model (Chen et al., 2007). Most studies of mTOR in TBI focus on its negative role in neuronal survival within hours of initial injury (Ding et al., 2015). Genetically constitutive mTOR activation worsened neurological scores, while inhibition by rapamycin benefits acute cognitive function improvement (Rozas et al., 2015, Nikolaeva et al., 2015, Park et al., 2012). However, the conclusion is debatable, since another study proved rapamycin, on the contrary, worsens cognitive function (Zhu et al., 2014). My current study also observed increased mTOR activation in the hippocampus after TBI, while by combining specific cell type markers, I identified mTOR activation not only in the mature neurons, astrocytes, and microglia but also in the NSCs, all at different time points (Figure 1.4 B). In chapter 3, I demonstrated that

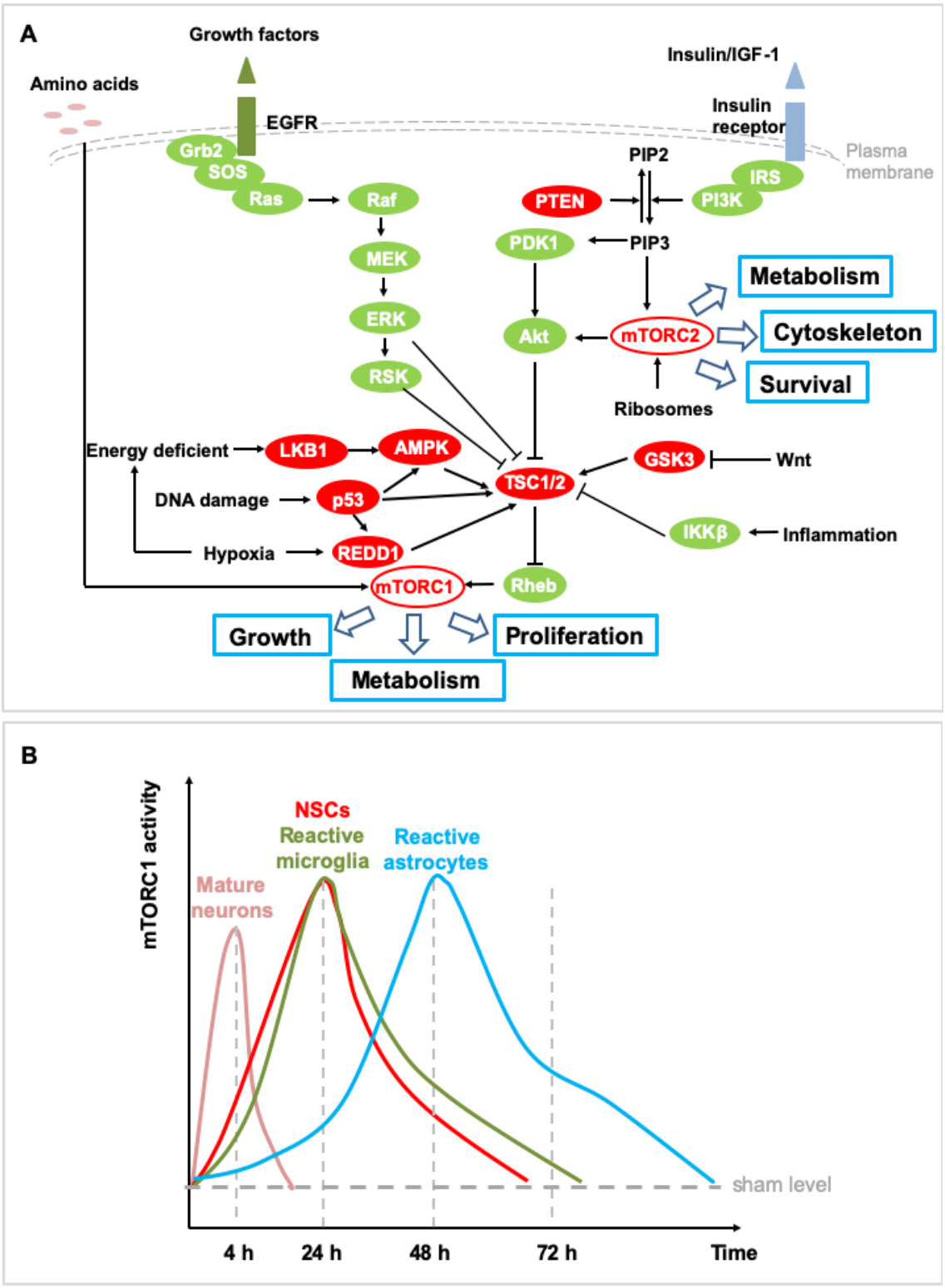


Figure 1.4: Signaling pathway of mTOR and mTORC1 activation in different cell types in the hippocampus after TBI.

(A) Signaling pathways regulate mTOR activities and roles of mTORC1 and mTORC2. Modified from Figure 2 (Laplante and Sabatini, 2012) and Figure 2 (Meng et al., 2018). (B) Activation of mTORC1 in different cell types at different time points after TBI.

rapamycin administration abolished NSC proliferation promoted by the injury, indicating besides neuronal survival, mTOR is also important in the innate repair mechanism activation (Wang et al., 2016b). It is speculated that mTOR activation in different cell types correlates with different functional relevance, which peaks at different time points as well. Thus, careful characterization of mTOR functions and identification of a time course of mTOR activation in individual cell types may provide more detailed information on whether mTOR activation as well as when and where mTOR activation is beneficial for functional recovery from TBI.

Taken together, agreements and debates co-exist in the mechanistic studies of neurogenesis after TBI, while some insights still be can obtained on the development of potential therapeutic approach from current knowledge. Although neurotrophic and growth factors have proven to be beneficial to enhance endogenous neurogenesis and improve functional recovery, the administration of polypeptide is either invasive or blocked by blood-brain-barrier. More issues include potential immune response to exogenous polypeptide and high cost for polypeptide production and storage. Development of small molecules mimicking neurotrophic and growth factor effects is an alternative and already has some promising results published. Other options include targeting primary pathways involved in neurogenesis in the injury environment. In chapter 4, I tested a small molecule targeting mTOR signaling and showed improved spatial learning function. Nevertheless, more investigations are needed to fully understand the molecular mechanism regulating the functional outcomes before it can provide feasible avenues for clinical applications.

Current therapeutic interventions and potential clinical applications

Based on current knowledge of various mechanisms mediating TBI induced neurogenesis, several reagents have been tested in animal models to gain information on their therapeutic potential and rationale for clinical trial testing. Erythropoietin (EPO), as a kidney originated cytokine, classically is considered as modulator of red blood cell production. Recently, it is found in the brain and exhibits neuroprotective roles (Nguyen et al., 2014). The administration of EPO in TBI animals increased neurogenesis and improved neurobehavioral outcomes (Lu et al., 2005, Xiong et al., 2009). EPO-mediated effects are linked to VEGF expression and VEGF receptor type 2 activation (Xiong et al., 2011b). Since EPO is already an FDA-approved and commercialized drug against anemia, Dr. Roberson's group conducted a randomized phase 2 trial in 200 closed head TBI patients. They found that an EPO regimen (500 IU/kg, intravenous infusion within 6 hours of initial injury with/without 2 additional doses every 24 h) failed to improve neurological outcomes by 20% and demonstrated the futility of conducting a further phase 3 trial with this regimen (Robertson et al., 2014). In addition, a recent multicenter trial of a different regimen (40,000 IU, subcutaneously, once per week up to 3 doses) failed to improve functional recovery again (Nichol et al., 2015). Failure of these individual regimens does not necessarily indicate the end of EPO application in TBI. Although species difference between human and rodents may impede the translation of results from animal experiments, treatment time, dose, and duration can be finely modified to achieve better outcomes in clinical trials in the future. Besides EPO,

no other reagent has been tested in TBI clinical trials in terms of enhancing neurogenesis. However, there are a few candidates available.

Cerebrolysin, a mixer of neuropeptides derived from purified brain proteins, has also displayed neurogenic effects in rodents by increasing mature neuron production and cognitive function improvement after TBI (Zhang et al., 2013b, Zhang et al., 2015b). Additionally, it has been tested in several clinical trials against stroke, Alzheimer's disease and TBI, most of which exhibited beneficial outcomes and stable safety in human subjects (Heiss et al., 2012, Chen et al., 2013, Gauthier et al., 2015, Thome and Doppler, 2012). Current phase 2 trial results suggest the necessity of phase 3 trial to step forward towards an effective treatment for TBI.

Statin, a cholesterol-lowering agent widely used in cardiovascular diseases, recently has been applied after TBI in mice as well. Its beneficial effects showed in various respects including increased neurogenesis in the dentate gyrus, reduced neuronal death in the hippocampal CA3 region, decreased glial response and pro-inflammatory cytokine in the lesion site, up-regulated VEGF and BDNF expression and improved neurological outcomes (Li et al., 2009, Wu et al., 2012, Wu et al., 2008, Lu et al., 2007). Imipramine, an antidepressant, has also shown to increase neurogenesis in the hippocampus and enhance cognitive function in mice model (Han et al., 2011). Recombinant tissue plasminogen activator (tPA) is an effective reagent against ischemic stroke, as it mainly resolves blood clots. Its application in TBI at subacute but not acute phase in rodents has led to increased generation of post-injury born

mature neurons along with cognitive functional recovery (Meng et al., 2014). These above reagents, as FDA-approved drugs, are all good candidates for clinical trials in human patients, since no more safety tests are required. Fine-tuning design of the regimen still needs further development and testing. For instance, the adverse effect of tPA on blood coagulation restricts its application only after hemorrhaging stops. The precise time point of drug administration in potential clinical trials should be designed accurately according to human TBI pathophysiological characteristics.

Some other novel molecule treatments aimed at neurogenesis tested in animal models include LM11A-31, thymosin β4 (Tβ4), 7,8-dihydroxyflacvone (DHF), angiotensin receptor 2 (AT_2) agonists, mesenchymal stromal cells (MSC)-derived exosomes, MLC901 and P7C3-A20. LM11A-31 is a small molecule of p75 neurotrophic receptor agonist, which was developed by Dr. Longo in 2006 and succeeded phase 1 safety trial conducted by PharmatrophiX in human subjects (Massa et al., 2006). Although initially designed for Alzheimer's disease, LM11A-31 has shown therapeutic effects in spinal cord injury, as well as in TBI. In TBI animals, LM11A-31 administration decreased cell death, increased proliferation of progenitors, enhanced neurogenesis, and improved spatial learning and memory impairments (Shi et al., 2013). Tβ4 is an actin-sequester protein, but also holds multiple other biological properties and has been applied to clinical trials in healthy volunteers to test safety and against venous ulcers (Crockford et al., 2010, Guarnera et al., 2007, Ruff et al., 2010). Treatment with Tβ4 in TBI mice reduced tissue lesion volume, increased neurogenesis in the

hippocampus and improved functional recovery, which indicated potential application of Tβ4 in TBI trials (Xiong et al., 2011a, Ye et al., 2013). These two reagents are candidates developed by biomedical companies. If proven to be effective in clinical trials, they are close to be commercialized in the field of brain trauma.

DHF is a natural small molecule activating TrkB receptor and mimicking BDNF effects. My colleagues and others reported neuroprotective functions of DHF treatment after TBI in animal models shown as decreasing neuronal death, reducing tissue lesion, and ameliorating dendritic degeneration (Wu et al., 2014, Chen et al., 2015, Zhao et al., 2015a, Agrawal et al., 2015). My colleagues also demonstrated beneficial effect of DHF on neurogenesis in the hippocampus after TBI, indicating DHF is a promising therapeutics aimed at neuroprotection and neuroregeneration at the same time (Zhao et al., 2016). Angiotensin, a hormone mainly controlling blood pressure through vasoconstriction, also has receptors expression in the brain. Activation of AT_2 mediated effects are called protective arm in brain injuries (Namsolleck et al., 2014). Thus, a small molecule AT_2 agonist, CPG42112A has been introduced in TBI mice, and neurogenesis was elevated at 35 days after a 3-day treatment, as well as improved neurobehavioral outcome (Umschweif et al., 2014).

Exosomes are tiny membrane vesicles secreted by several cell types to the body fluids, and normally contain proteins, mRNAs, and miRNAs from parent cells (Ching and Kingham, 2015). Their applications as diagnostic markers and therapeutic targets have been widely studied in cancer biology and emerged in

the field of neuroregeneration (Tominaga et al., 2015, Ching and Kingham, 2015). MSC-derived exosomes delivery to TBI rats has improved functional recovery, increased angiogenesis and neurogenesis as well as deceased gliogenesis and inflammation (Zhang et al., 2015a).

MLC901, the active component in a traditional Chinese medicine, showed neuroprotective effects when applied at 2 h after injury and enhanced neurogenesis if introduced in drinking water during the first week of injury (Quintard et al., 2014). P7C3-A20 is a compound related to latrepirdine, a drug that failed in Pfizer and Medivation conducted phase 3 clinical trials against Alzheimer's disease and Huntington's disease. The new compound exhibits neuroprotective potential, so it was applied to TBI rats to test whether benefits in functional outcomes can be achieved. By an initial dose at 30 min after FPI with concessive doses twice per day for 7 days, P7C3-A20 successfully reduced contusion volume, increased cell proliferation in the first week, enhanced generation of mature neurons, and improved spatial learning and memory performance in Morris Water Maze test (Blaya et al., 2014). These novel approaches are still in the early phases of development and lack of pharmacokinetics and toxicity analysis. In addition, more animal studies are in need to validate these potentially exciting therapeutic approaches.

Although it seems that more than enough promising candidates for TBI treatment exit, none of them have succeeded in clinical trials. Before an authentic cure is translated from animal experiments to patients' bedside, well-designed investigations should be conducted on the molecular mechanisms of the effective

reagent. To establish clinical relevance, a detailed comparison between animal and human pharmacokinetics should be assessed. If these details can be analyzed thoroughly, fine-tuning strategies can be developed to achieve high efficacy and strong potency and to maximize therapeutic benefits for TBI patients.

Summary

As a promising therapeutic target for TBI patients, great efforts have been spent on endogenous hippocampal NSC mediated post-traumatic neurogenesis. Extensive information has been obtained regarding the phenomenon of TBI-enhanced NSC proliferation, properties of post-injury born neurons, and functional correlation between post-traumatic neurogenesis and functional recovery. A few clinical trials are undergoing and targeting post-traumatic neurogenesis with the hope of achieving better cognitive functional outcomes. Whereas, more questions are yet to be answered.

The following chapters will describe my work in assessing if TBI severity affects the neurogenesis level and if post-injury born neurons can integrate anatomically and functionally. The molecular mechanism of TBI-enhanced NSC proliferation was investigated. In addition, a pharmacological approach was applied to target the mechanism, and spatial learning and memory function was examined.

CHAPTER 2

CHARACTERIZATIONS ON TBI RESHAPED NEUROGENESIS REGARDING POST-TRAUMATIC NSC PROLIFERATION, POST-INJURY BORN IMMARUTE NEURON PRODUCTION, AND POST-INJURY BORN MATURE NEURON GENERATION AND INTEGRATION

Hypotheses

1) TBI reshapes neurogenesis by affecting NSC proliferation, post-injury born immature neuron production, and post-injury born mature neuron generation in a severity-dependent manner, and 2) TBI alters post-injury born neurons' anatomical integration in the neural network, and promotes their functional recruitment in standard housing environment and upon hippocampus-dependent behavior recruitment.

Introduction

TBI causes a wide range of complicated pathological changes, among which neuronal death and degeneration directly contribute to functional deficits (Lu et al., 2005, Hall et al., 2008). Diffusive injury after trauma affects much larger brain regions than the initial impact site, and the hippocampus is among the most vulnerable areas to neuronal death (McIntosh et al., 1998, Ariza et al., 2006). Consequently, learning and memory deficit is one of the most commonly complained symptoms among TBI patients (Wolf and Koch, 2016). Since no FDA-approved drug is available to stop cell death, it is urgently desired to

develop an alternative approach aiming to generate new neurons and compensate for cell loss.

Neurogenesis has been widely detected in the adult hippocampus in songbirds, rodents, primates, and humans (Cameron and McKay, 2001, Eriksson et al., 1998, Kempermann and Gage, 2000, Kornack and Rakic, 1999, Kuhn et al., 1996, Leuner et al., 2007, Burd and Nottebohm, 1985). NSCs in the SGZ undergo constant activation, proliferation, neuronal differentiation, and maturation, which eventually generate new functional granule neurons (Christian et al., 2014, Shapiro and Ribak, 2005, Zhao et al., 2006). Adult NSC-derived neurogenesis provides a potential resource for neuronal replenishment to the injured hippocampus following TBI. Post-traumatic neurogenesis has been reported responsible for spontaneous recovery (Schmidt et al., 1999, Blaiss et al., 2011, Sun et al., 2015), since enhanced endogenous neurogenesis achieved by introduction of neurogenic agents is positively correlated with functional improvements in the injured animals (Lu et al., 2003, Kleindienst et al., 2005, Lu et al., 2005, Wu et al., 2008). Together, it is feasible to promote compensation on neuronal loss by enhancing NSC-mediated endogenous neurogenesis, and consequently to rebuild damaged circuits and restore cognitive functions.

Enormous studies have reported TBI induced cell proliferation, which includes NSC proliferation (Braun et al., 2002, Chirumamilla et al., 2002, Dash et al., 2001, Kernie et al., 2001, Ramaswamy et al., 2005, Rice et al., 2003, Rola et al., 2006, Sun et al., 2005, Sun et al., 2007, Yoshimura et al., 2003, Gao and Chen, 2013, Gao et al., 2009a, Bye et al., 2011), however, the results on post-

traumatic neurogenesis are controversial. Increased (Sun et al., 2005, Sun et al., 2007, Braun et al., 2002), unchanged (Chirumamilla et al., 2002, Bye et al., 2011, Gao and Chen, 2013), and decreased (Rola et al., 2006) post-traumatic neurogenesis have all been described. Since different TBI models were used in individual studies: fluid percussion injury (FPI) model (Chirumamilla et al., 2002, Rice et al., 2003, Sun et al., 2005, Sun et al., 2007), controlled cortical injury (CCI) model (Braun et al., 2002, Rola et al., 2006, Gao and Chen, 2013), cortical contusion model (Braun et al., 2002), and impact-acceleration model (Bye et al., 2011), severities of the injuries were not comparable. Thus, I speculated that injury severity is the key variable that causes the contradictory results and hypothesized that TBI severity affects neurogenesis level. To test my hypothesis, I introduced mild, moderate, and severe injuries to adult mice by a CCI model, and evaluated NSC proliferation, immature neuron number, and post-injury born mature neuron number. These findings are the first to elucidate that TBI reshapes post-traumatic neurogenesis in a severity-dependent manner and partially explains the pre-existing controversy.

Besides compensating the amount of cell loss on a population level, post-injury born neurons are supposed to undergo morphological and functional maturation before they can contribute to neurological behaviors. Although individual post-injury born neurons have shown comparable electrophysiological properties with counterparts in sham animals, they have morphologically shown altered dendritic arborization and ectopic migration (Villasana et al., 2015, Ibrahim et al., 2016), indicating possible circuit integration alteration. Moreover,

previous studies using retrograde tracer Fluorogold labeling showed tracer uptake by post-injury born neurons from the injection site in the CA3 region. However, this phenomenon only proves axonal extension to CA3, but is not sufficient to prove functional synapse formation between post-injury born neuron and CA3 neurons (Emery et al., 2005, Sun et al., 2007). Together, it is not clearly studied if TBI affects the pattern of anatomical integration of post-injury born neurons, and if they were exerting neuronal activity in standard housing environment and upon behavioral recruitment. I hypothesized that TBI alters anatomical integration of post-injury born neurons and promotes their functional integration. To test the hypothesis, I utilized a dual-virus pseudotyped rabies virus monosynaptic tracing system to label pre-synaptic targets of post-injury born neurons, and used c-fos, an immediate early gene (IEG) (Ramirez et al., 2013, Garner et al., 2012), as a neuronal activation marker to evaluate functional integration of post-injury born neurons in standard housing environment and upon behavior task. My results proved that post-injury born neurons form synapses with their normal distal (LEC/PRH) and local (granule neurons) targets. However, they are also innervated by the medial habenular nucleus and retrosplenial granular cortex neurons and CA1 neurons, which has not been reported before. TBI promotes functional recruitment of post-injury born neurons in both standard housing environment and upon spatial learning and memory task. To my knowledge, these findings are the first to describe the influences of TBI on post-injury born neuron integration pattern.

Materials and methods

Animal care

Male C57 BL/6 mice were purchased from Envigo and Jackson Laboratories and housed in a 12/12-hr light/dark environment. Food and water were given ad libitum. Nestin-GFP (green fluorescent protein) transgenic mice were a kind gift of Dr. Enikolopov at Cold Spring Harbor Laboratories (Mignone et al., 2004). All procedures were performed under protocols upon approval from Indiana University's Animal Care and Use Committee.

Controlled cortical impact traumatic brain injury

Male C57 BL/6 mice were used at the age of 8 to 10 weeks (n=76). In the studies, mice were subjected to CCI injury like previously described (Gao and Chen, 2013, Gao and Chen, 2011). Briefly, mice were anesthetized using a solution of 2.5% Avertin (Sigma, St. Louis, MO) followed by fixation in a stereotaxic frame (Kopf Instruments,Tujunga, CA). Under sterile procedures, the skin was retracted to expose the skull. A 4 mm craniotomy was performed laterally midway between the central suture and the temporalis muscle, posteriorly midway between the bregma and lamda sutures. The skullcap was carefully removed, leaving dura undisrupted. The impactor tip used in the experiment has a diameter of 3.5 mm. The tip was angled perpendicular to the exposed cortical surface when performing injury.

The mouse CCI injury impactor was controlled electromagnetically, which allows independent adjustments of contact velocity, contact duration, and

deformation depth, respectively. In the severity experiments, contact velocity was constantly set at 3.5 m/s, impact duration was constantly set at 0.1 s, and deformation depth was the parameter that determines injury severity. It was set at 0.2 mm, 1.0 mm, and 1.2 mm to generate mild, moderate, and severe TBI, respectively. In the presynaptic neuron tracing studies and the functional integration studies, the parameters were set as 3.5 m/s, 0.1 s, and 1.0 mm. The injury site was sutured after bleeding stops. The animals were put on a heating pad set at 36–37 °C to maintain body temperature the whole time during surgery and recovery.

BrdU injection

To evaluate cell proliferation, BrdU was delivered at 44 h following TBI (100 mg/kg in saline, intraperitoneally [i.p.]; Sigma, St. Louis, MO). To quantify post-injury born mature neurons, BrdU was injected daily in the first week following TBI (50 mg/kg in saline, i.p.). To label post-injury born neurons and study their neuronal activity, BrdU was injected daily for the first two weeks after TBI (50 mg/kg in saline, i.p.).

Virus production and injection

To trace pre-synaptic neurons innervating post-injury born neurons, a dual-virus pseudotyped rabies virus monosynaptic tracing system was used (Callaway and Luo, 2015). Briefly, the genome of rabies virus was engineered by replacing its own glycoprotein gene with a fluorescent protein gene. The engineered rabies

virus was then pseudotyped with envelope protein A (EnvA) from avian sarcoma and leukosis virus (ASLV), which specifically binds and infects cells expressing a TVA (tumor virus A) receptor. A helper virus was used to express TVA receptor and rabies glycoprotein for the pseudotyped rabies virus infection, and its packaging and retrograde trans-synaptic tracing, respectively. In my studies, EnvA G-Deleted Rabies-dsRedExpress pseudotyped rabies virus was purchased from the GT3 Core Facility of the Salk Institute for Biological Studies (La Jolla, CA). The helper virus constructs were RV-Syn-GTRgp (a retrovirus construct), a kind gift of Dr. Fred Gage from the Salk Institute of Biological Studies (Vivar et al., 2012). The retroviral helper virus was packaged with plasmids expressing vesicular stomatitis virus G glycoprotein (VSVG), Gal, and Pol. Virus packaging was conducted by Eric Lee Thompson in the Department of Pharmacology and Toxicology at Indiana University School of Medicine.

To trace the pre-synaptic target of post-injury born neurons, intracranial injections were performed at two sites with retroviral helper virus at the following coordinates: AP -1.8 mm, LM -1.5 mm, depth -1.6 mm, and AP -2.4 mm, LM -1.8 mm, depth -1.6 mm. Each site was injected with 1 ul retrovirus. Pseudotyped rabies virus was then injected at the same sites with 1 ul for each site.

Tissue processing

Animals in each experiment were sacrificed at time points specified in figures. Briefly, the animals were deeply anesthetized a solution of 2.5% avertin. Transcardial perfusion was performed with cold saline first, followed by 4%

paraformaldehyde (PFA) in PBS. The brains were harvested and post-fixed overnight in PFA at 4°C. Then the brains were transferred to 30% sucrose for 48h at 4°C for cryoprotection. Serial coronal sections were cut at 30 um thick using a cryostat (LeicaCM 1950), followed by storage at -20 °C. For pre-synaptic neuron tracing studies, serial horizontal sections were cut at 30um thick using the cryostat, followed by storage at -20°C.

Histology and immunohistochemistry

Nissl staining was used to analyze anatomical changes. Briefly, sections were successively incubated in a solution of 0.1% cresyl violet (Sigma) for 20 min, quickly rinsed in distilled water, differentiated in 95% ethanol for 3 min, dehydrated in 100% ethanol for 5 min twice, cleared in xylene for 5 min twice, air-dried, and mounted with DPX (Sigma).

FJB staining was used to detect dying neurons. Briefly, brain sections were hydrated in distilled water for 5 min, followed by 20 min incubation in a solution containing 0.06% potassium permanganate (Sigma) at room temperature. The sections were successively washed in distilled water for 5 min twice, incubated in a 0.0004% solution of FJB (Sigma) for 20 min, washed again in distilled water for 3 times, incubated in a solution of 0.01% 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) for 10 min, air-dried, and mounted with DPX.

Immunostaining was performed to specify target cells. Briefly, free-floating sections were washed at room temperature in PBS for 3 times, incubated with blocking buffer containing 1% bovine serum albumin, 0.1% Triton X-100, 5%

normal goat serum in PBS at room temperature for 1 h. Then they were incubated in primary antibody at 4 °C overnight. Then sections were washed in PBS for 3 times, incubated with secondary antibody for 2 h at room temperature, and followed by DAPI treatment for 2 min. The sections were washed in PBS for 3 times and mounted with Fluoromount-G solution (SouthernBiotech). For BrdU detection, HCl pretreatment was performed prior to standard staining protocol. Briefly, sections were treated with 2N HCl for 1 h at room temperature, neutralized with 0.1M borate buffer (pH 8.4) for 10 min, washed in PBS 3 times, and processed to standard blocking protocol. The primary antibodies and final concentrations used were listed in Table 2. Jackson ImmunoResearch Laboratories, Inc. secondary antibodies were applied in a dilution of 1:1000.

Cortical cavity volume measurement

Series of every sixth sections (30 μm thick, 180 μm apart) were processed to Nissl staining to show the spared cortex. Images were acquired with a Zeiss microspore. The contours of the contralateral and ipsilateral spared cortices were drawn in the sections that cover the injured cortex, and contours enclosed volume was measured in AxioVision Rel 4.8 software (Zeiss). The percentage cortex of the cavity was calculated by the following formula: percentage of the cortical cavity = (contralateral cortex volume – ipsilateral spared cortex volume) / contralateral cortex volume × 100%.

Table 2: Antibodies used in the studies.

Antibody	Company	Catalog Number	Dilution
Mouse anti- β-actin	Abcam	ab8226	1:10000
Rat anti-BrdU	Abcam	ab6326	1:200
Rabbit anti-c-fos	Santa Cruz	sc52	1:200
Rabbit anti-CD11b	Abcam	ab133357	1:200
Guinea pig anti-DCX	Millipore	AB2253	1:500
Chicken anti-GFP	Abcam	ab13970	1:1000
Mouse anti-GFAP	Sigma	G3893	1:800
Goat anti-MCM2	Santa Cruz	sc-9839	1:200
Mouse anti-NeuN	Millipore	MAB377	1:500
Rabbit anti-NG2	Millipore	AB5320	1:200
Rabbit anti-pS6	Cell Signaling	4858	1:200
Rabbit anti-RFP	Invitrogen	R10367	1:1000
Goat anti-Sox2	R&D	AF2018	1:1000
Rabbit anti-Sox2	Abcam	ab97959	1:1000
Rabbit anti-S6	Cell Signaling	2217	1:1000

Cell counting

An inverted fluorescent microscope system (Zeiss Axiovert 200 M) was used to analyze immunostained sections. The total number of target cells was determined by counting every individual positive cell (even partial positive cell at the borders of sections) in multiplanes throughout the entire interested region in every section, and the number from each section analyzed was added together. For BrdU labeled cell proliferation, cell fate tracing, and post-injury born neuron activity, a series of every sixth section (30 μm thick, 180 μm apart, 12 to 16 sections for each animal) covering the whole hippocampus were processed with immunostaining. To quantify immature neurons, 3 epicenter sections (30 μm thick, 180 μm apart) were processed with immunostaining. To analyze pre-synaptic neurons of post-injury born neurons, sections covering the whole brain were processed to immunostaining.

To calculate the cell density of target cells, the volume of the granule cell layer of the dentate gyrus area was measured by creating contours by Zeiss software (AxioVision v4.8). To evaluate NSC proliferation, cell density was calculated by dividing the total cell number by the volume of the granule cell layer. For surviving proliferated cells and newly generated mature neurons assessment, cell density was calculated by dividing the total number by the volume of the dentate gyrus. For immature neuron quantification, cell density was calculated by dividing the total number by the volume of the granule cell layer.

In order to assess ratios of mature neurons versus reactive glia in surviving proliferated cells, epicentral sections from each animal were stained

with antibodies against BrdU and GFAP or NG2 or CD11b, respectively. BrdU single positive cells and target double positive cells were counted. The percentage of target cell type was respectively calculated. The ratio of each cell type in the BrdU positive population was then normalized to match 100% in total.

Microscopy

The inverted microscope system (Zeiss Axiovert 200 M) used in the experiments was equipped with an apotome for optical sectioning and a motorized stage for stitched images and interfaced with a digital camera (Zeiss Axio Cam MRc5). After image acquisition, the images were assembled and labeled by Photoshop 7.0 (Adobe System).

Statistical analysis

Quantitative data were displayed as average ± standard deviation. Results were analyzed by one-way ANOVA. The *post hoc* analysis was done via Tukey's honest significance test using SPSS software. Functional integration data was analyzed via two-tailed student's t-test. Significance was set at $p<0.05$.

Results

CCI model induced different severities of TBI

TBI, as a complex disease, ranges from mild to severe levels. To generate different injury severities in mice, a CCI model was used as previously described (Gao and Chen, 2013, Gao and Chen, 2011) and injury levels were managed by

deformation depths set at 0.2 mm, 1.0 mm, and 1.2 mm, corresponding to mild, moderate, and severe injury, respectively. Injury severity was further confirmed by histological examination (Figure 2.1). Consistent with previous reports from my colleagues, mild injury caused limited cortical disruptions without obvious cavity formation (lesion volume at 0.2% ± 5.4%, p=1, Figure 2.1 B and E) (Gao and Chen, 2011). Moderate TBI induced a cortical cavity (cavity volume is 14.3% ± 1.0%, p=0.001, Figure 2.1 C and E) with an intact corpus callosum (Gao et al., 2008), while severe TBI dramatically increased cavity volume (26.2% ± 1.8%, p<0.001, Figure 2.1 D and E), further destroyed the corpus callosum and even deformed the hippocampal structure.

At the cellular level, I evaluated cell death in the hippocampal dentate gyrus (HDG), where the NSCs reside, with Fluoro-Jade B (FJB) staining. FJB-positive cells were hardly observed in the HDG of the mouse brain that received sham (Figure 2.2 A) or mild CCI injury (Figure 2.2 B). FJB-positive cells were vastly detectable in the HDG of mouse brain that received moderate CCI injury (Figure 2.2 C), mainly at the inner 1/3 of the granular cell layer, where the immature neurons locate. My colleagues previously reported that most of the dead cells in the HDG following moderate CCI-injury are immature neurons (Gao et al., 2008). As the injury severity escalated to the severe level, the FJB-positive cells not only located at the inner 1/3 of the granular cell layer, they also were observed in the outer granular cell layer and hilus (Figure 2.2 D), where mature granular neurons and interneurons respectively locate. Immunostaining results confirmed that the majority of cells dying in severe TBI were co-stained with

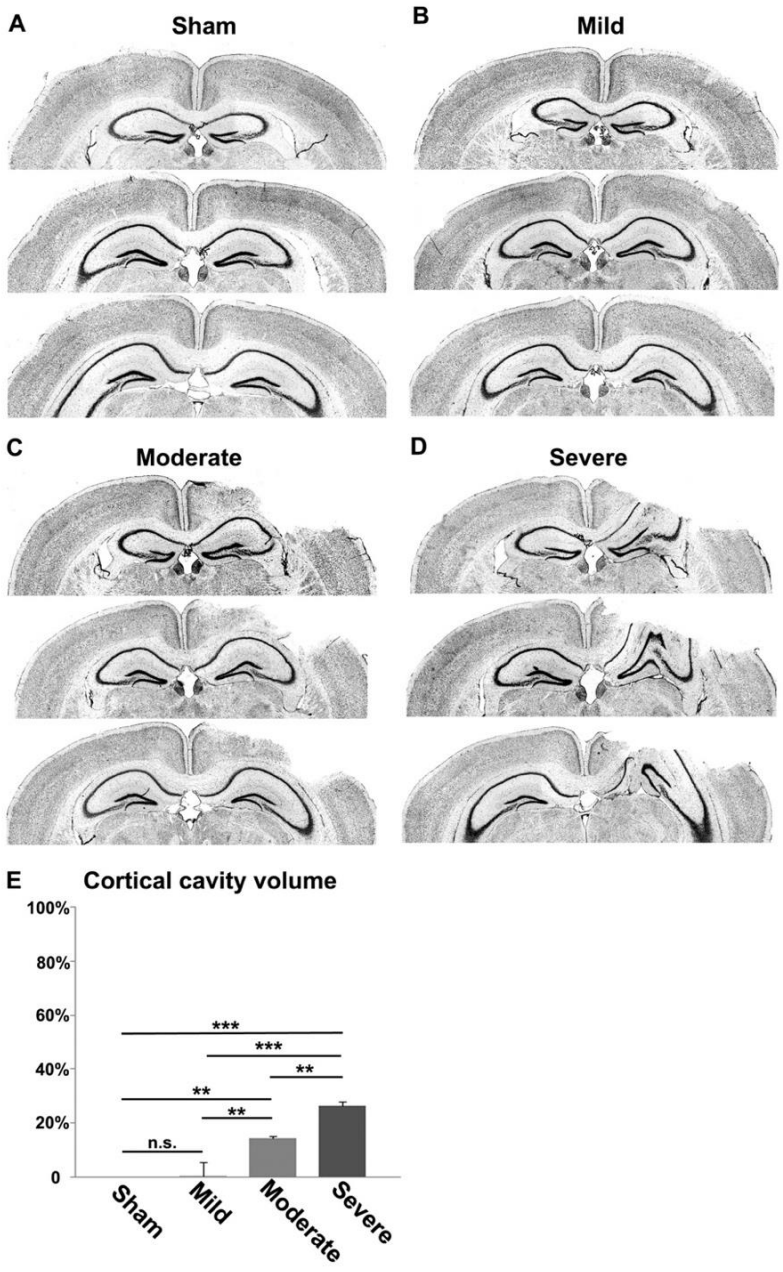


Figure 2.1: TBI severities shown by Nissl staining.

Brain tissues collected at 48 h after traumatic brain injury (TBI). (A-D) Nissl staining of serial coronal sections of sham (A), mild (B), moderate (C), and severe TBI (D) mice shows the characteristic structures in different severities of TBI. (E) Quantification of cortical cavity volume in different severities. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. not significant, n=3 for each group).

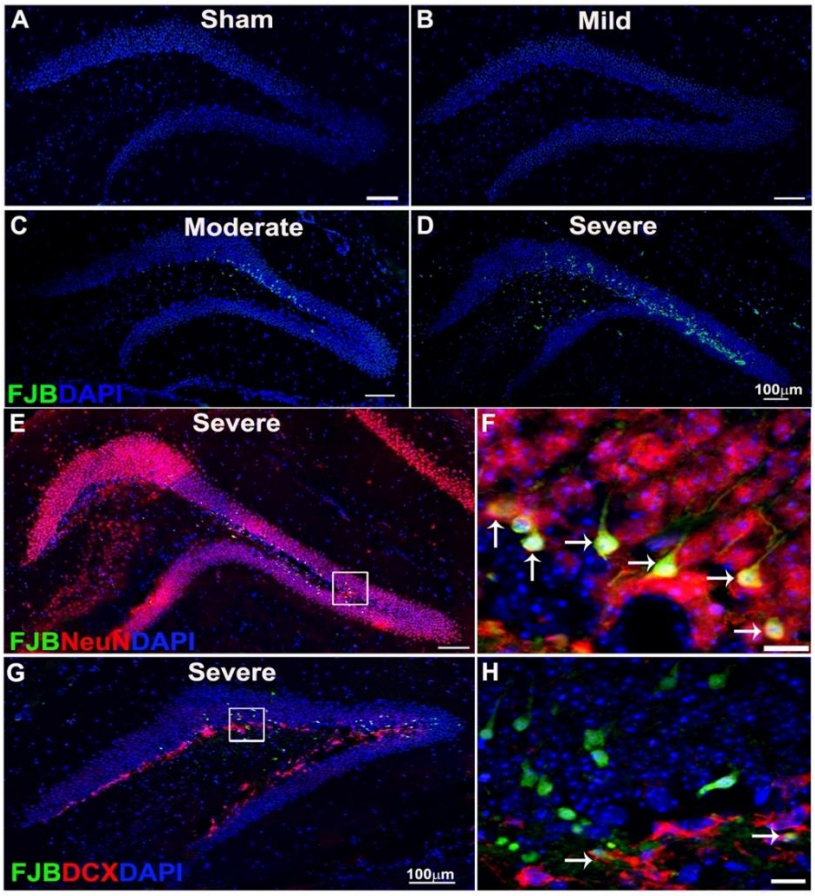


Figure 2.2: Severe TBI mainly causes mature neuron death.

Brain tissues collected at 24 h after traumatic brain injury (TBI). (A-D) Fluoro-Jade B (FJB) staining shows dying cells in the hippocampal dentate gyrus (HDG) of sham (A), mild (B), moderate (C), and severe TBI (D) mice. (E, F) Immunostaining against NeuN (red) for mature neurons followed by FJB staining (green) for dying cells. (G, H) Immunostaining against doublecortin (DCX; red) for immature neurons followed by FJB staining (green) for dying cells. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows HDG structure. (F, H) Enlarged images of E, G (indicated by white boxes) to show co-staining of NeuN and FJB (F, indicated by white arrows) and co-staining of DCX and FJB (H, indicated by white arrows).

Neuronal Nuclei (NeuN) positive mature neurons (Figure 2.2 E and F, indicated by white arrows) and a small proportion were co-labeled with doublecortin (DCX) positive immature neurons (Figure 2.2 G and H, indicated by white arrows).

TBI severity affects the survival of proliferated cells in the HDG

The effect of TBI on overall cell proliferation in the HDG during the first week post-trauma has been investigated widely in different models by BrdU incorporation (Braun et al., 2002, Chirumamilla et al., 2002, Dash et al., 2001, Kernie et al., 2001, Ramaswamy et al., 2005, Rola et al., 2006, Sun et al., 2005, Sun et al., 2007, Yoshimura et al., 2003, Gao and Chen, 2013, Gao et al., 2009a, Bye et al., 2011). Whether the severity of injury affects cell proliferation and the subsequent survival is unclear. To address this question, I injected animals with BrdU (50 mg/kg) intraperitoneally (i.p.) once per day for the first week following surgery to label the proliferating cells and collected tissues at 4 weeks after TBI (Figure 2.3 A). A series of every sixth section (12 to 16 sections in total for each animal), which covered the whole hippocampus, were stained for BrdU.

BrdU-positive cells in the HDG were quantified. Sham animals displayed the baseline of surviving proliferated cells in the HDG at 969 ± 175/mm^3 (Figure 2.3 B and F); mild injury animals had a similar level at 1267 ± 161/mm^3 ($p=0.985$, Figure 2.3 C and F). In moderate injury animals, the density of surviving proliferated cells was dramatically elevated to 3739 ± 1250/mm^3 ($p=0.039$, Figure 2.3 D and F). The number was further elevated to 5406 ± 1611/mm^3 ($p=0.001$, Figure 2.3 E and F) in severely injured animals. Collectively, moderate and

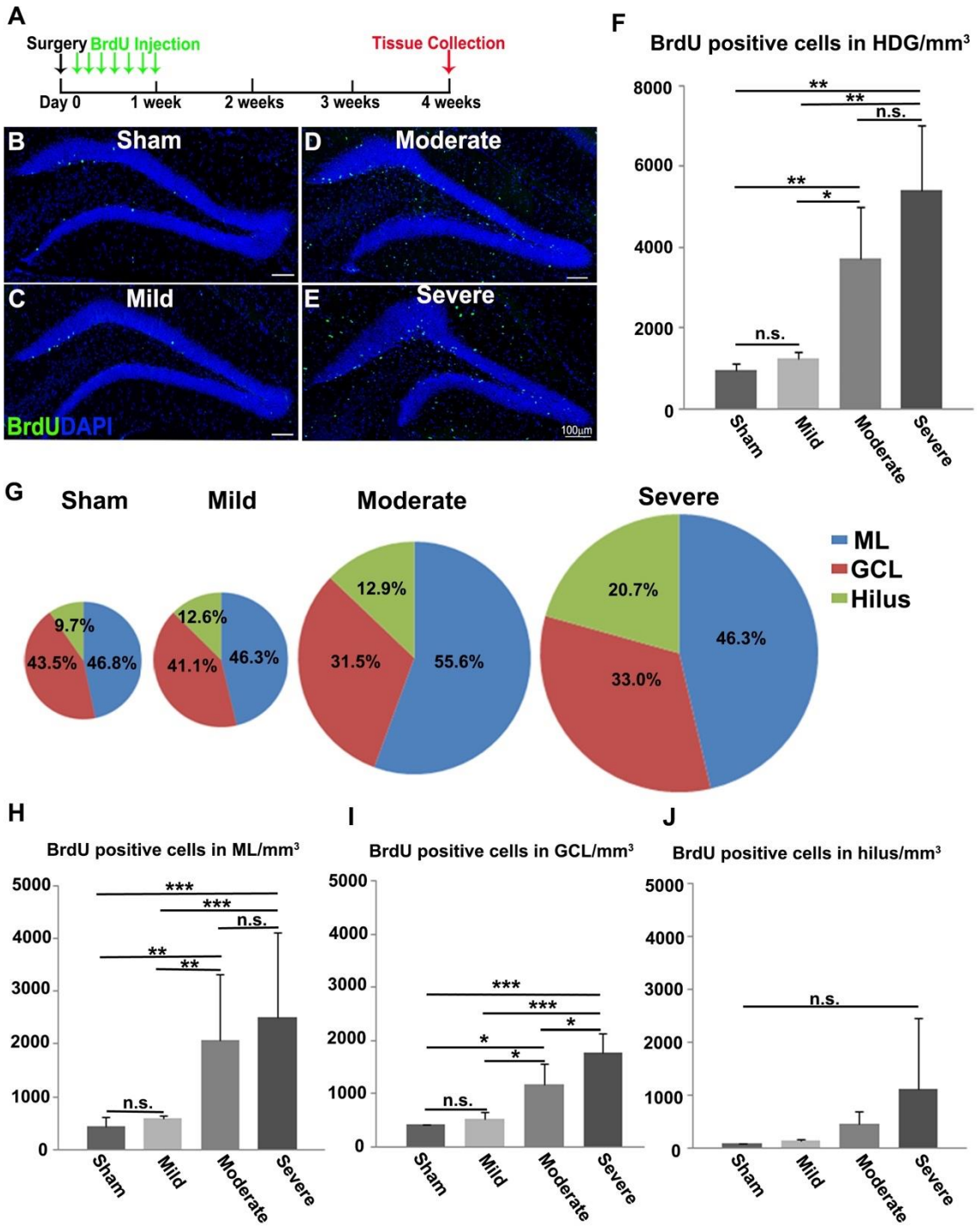


Figure 2.3: TBI severity affects surviving proliferated cells.

(A) Schematic shows experimental strategy. (B-E) Immunostaining with 5-bromo-2'-deoxyuridine (BrdU; green) to identify surviving proliferated cells in the

hippocampal dentate gyrus (HDG) in sham (B), mild traumatic brain injury (TBI) (C), moderate TBI (D), and severe TBI (E) mice. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows the HDG structure. (F) Quantification of BrdU-positive surviving proliferated cells in the HDG of mice after sham surgery and different TBI severities. (G) Ratio of BrdU-positive surviving proliferated cells in individual HDG subregions, molecular layer (ML), granule cell layer (GCL), and hilus, after sham surgery and different TBI severities. Pie chart sizes indicate the scale of total BrdU-positive cell numbers in different groups. (H-J) Quantification of BrdU-positive surviving proliferated cells in the ML, GCL, and hilus, respectively. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. not significant, n=3 for sham group, n=4 for mild and moderate TBI groups, and n=5 for severe TBI group).

severe, but not mild TBI, promoted the number of surviving proliferated cells at 4 weeks after injury.

TBI severity affects NSC proliferation

Various types of cells proliferate post-trauma in the HDG, including NSCs and reactive glia (Braun et al., 2002, Chirumamilla et al., 2002, Dash et al., 2001, Kernie et al., 2001, Ramaswamy et al., 2005, Rice et al., 2003, Rola et al., 2006, Sun et al., 2005, Sun et al., 2007, Yoshimura et al., 2003, Gao and Chen, 2013, Gao et al., 2009a). A previous study of my colleagues' in moderate TBI showed that injury transiently enhanced NSC proliferation, peaking at 44-48 h after initial injury (Gao et al., 2009a). To examine NSC proliferation after different levels of injuries, proliferating cells were pulse-labeled by BrdU (i.p., 100 mg/kg) at 44 h, and the brains were collected at 48 h. The cells that proliferated between 44-48 h following surgery were quantified (Figure 2.4 A). In the HDG of sham animals, a small number of cells undergoing proliferation were mainly observed in the SGZ, where the NSCs reside (Figure 2.4 B). Mild injured animals showed a similar number and distribution of BrdU-positive cells in the HDG (Figure 2.4 C). Moderate and severe TBI animals exhibited an obvious increase of BrdU-positive cells and also a dispersed distribution into the molecular layer and hilus (Figure 2.4 D and E).

It is known that post-traumatic cell proliferation also contains glial reactivation (Laird et al., 2008). To further discern NSC proliferation, I performed double immunostaining with antibodies against BrdU and transcription factor

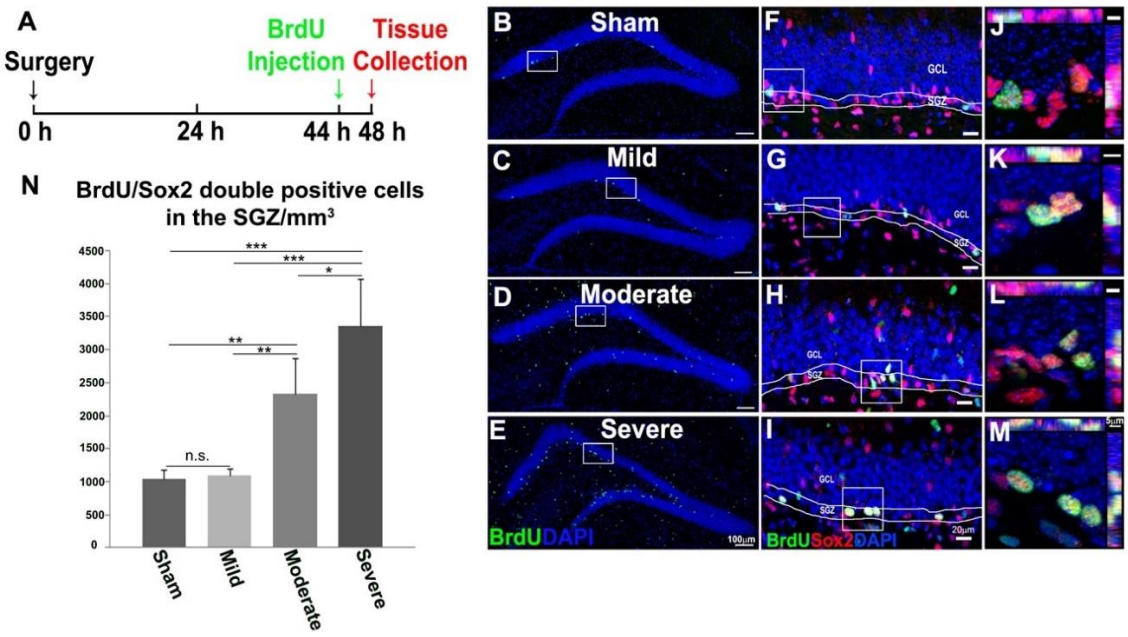


Figure 2.4: TBI severity affects NSC proliferation.

(A) Schematic shows experimental strategy. (B-E) Immunostaining with 5-bromo-2'-deoxyuridine (BrdU; green) to detect proliferating cells in sham (B), mild (C), moderate (D), and severe TBI (E) animals between 44-48 h after injury. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows hippocampal dentate gyrus (HDG) structure. (F-I) Double immunostaining against BrdU (green) and transcription factor Sox2 (red) to identify neural stem cell (NSC) proliferation in the subgranular zone (SGZ) of sham (F), mild (G), moderate (H), and severe TBI (I) mice. (J-M) 3-dimensional reconstruction of proliferating NSCs in F-I (indicated in white boxes) to show BrdU and Sox2 co-label. (N) Quantification of BrdU and Sox2 double-positive NSC proliferation in SGZ in sham and different TBI severities. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. not significant, n=5 for each group)

Sox2, since Sox2 is required for NSC self-renewal and widely used as an NSC marker (Graham et al., 2003, Favaro et al., 2009). Because hippocampal NSCs exclusively reside in the SGZ, proliferating NSCs were quantified based on their specific location within the SGZ and co-staining with Sox2 (Figure 2.4 F-M). In sham animals, I observed the baseline of NSC proliferation to be 1038 ± 135/mm^3 (Figure 2.4 F, J, and N), which represents normal adult neurogenesis in physiological conditions. In mild injury animals, NSC proliferation was not altered compared with sham animals (1096 ± 105 /mm^3, p=0.998, Figure 2.4 G, K, and N). After moderate TBI, NSC proliferation showed a significant 2.2-fold increase (2332 ± 540/mm^3, p=0.001, Figure 2.4 H, L, and N). This result is consistent with previous data (Gao and Chen, 2013). In severe injury animals, NSC proliferation was further boosted by 3.2-fold compared with sham animals (3352 ± 714/mm^3, p<0.001, Figure 2.4 I, M, and N).

Sox2 is also expressed in some of the reactive glia. Therefore, to rule out the possible interference from the reactive glia adjacent to the SGZ, I took advantage of a Nestin-GFP (green fluorescent protein) transgenic mouse line, in which GFP expression is driven by Nestin promoter and is observed in the NSCs but not in reactive glia (Mignone et al., 2004). With this unique mouse line, I validated that more than 83% of BrdU and Sox2 double-positive cells in the SGZ were GFP-positive NSCs following a moderate level of CCI-injury (Figure 2.5).

TBI severity affects immature neuron number

Previously, my colleagues have demonstrated that immature neurons are the most vulnerable cell type in the HDG following moderate TBI (Gao et al., 2008).

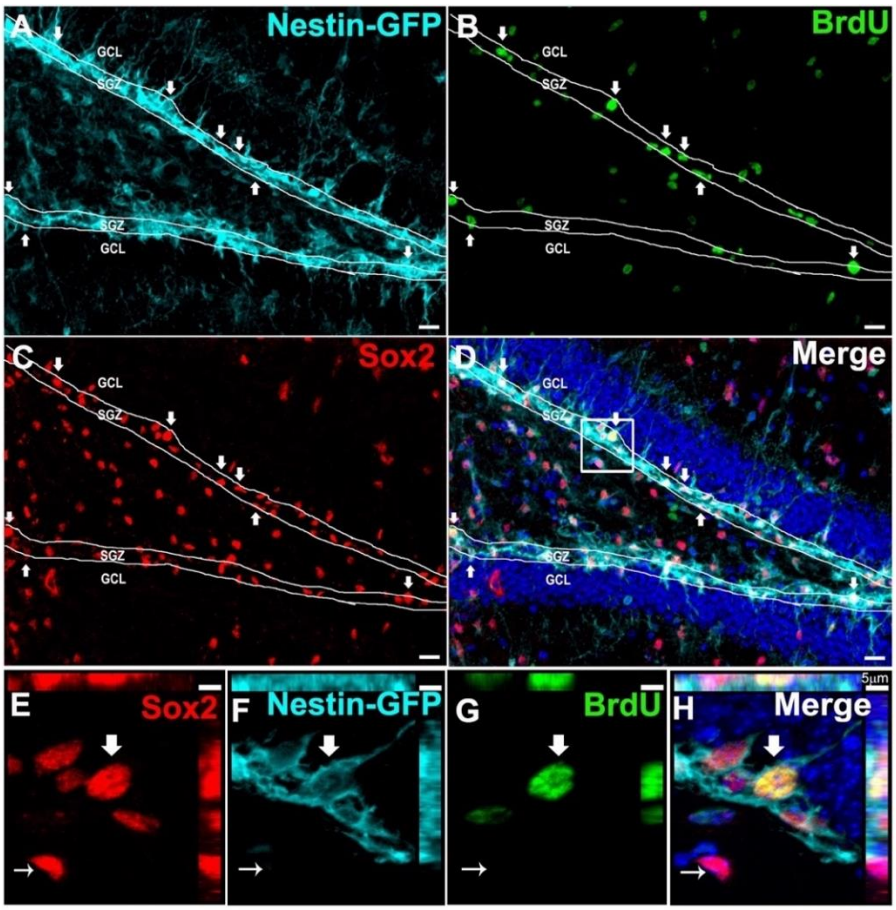


Figure 2.5: Validation of NSC proliferation following TBI with Nestin-GFP transgenic mice.

Brain was collected at 48 h after moderate traumatic brain injury (TBI) in Nestin-GFP mouse. (A-D) Immunostaining with green fluorescent protein (GFP), 5-bromo-2'-deoxyuridine (BrdU), and Sox2 confirms that majority of BrdU and Sox2 double-positive cells in the subgranular zone (SGZ) (indicated by white arrows) are GFP-positive neural stem cells. (E-H) 3-dimensional reconstruction of D (indicated in white box) shows co-label of Sox 2 and GFP in SGZ. A Sox2, GFP, and BrdU triple positive proliferating NSC in the SGZ was indicated by a white arrowhead. A Sox2 solely positive reactive glia in the hilus was indicated by a white arrow.

To assess the effects of injury severity on immature neuron number, I collected brains at 2 weeks post-injury at different severities and counted Doublecortin (DCX)-positive immature neurons in the HDG (Figure 2.6 A). Two weeks is the critical time point for a newly born and immature neuron to survive and differentiate into a mature neuron (Christian et al., 2014). In all the groups, DCX-positive cells resided primarily in the inner 1/3 of the granule cell layer (GCL) with their neurites growing towards the molecular layer (ML) (Figure 2.6 B-E). I then quantified DCX- positive cell numbers in each group (Figure 2.6 F-J). In sham animals, there were 23,432 ± 3,861/mm^3 DCX-positive cells residing in the GCL (Figure 2.6 F and J). No significant alteration was detected in the number of DCX-positive cells in the HDG following mild TBI (30,528 ± 6,495/mm^3, p=0.738, Figure 2.6 G and J) and moderate TBI (31,640 ± 5,778/mm^3, p=0.681, Figure 2.6 H and J). However, in severe injury animals, the number of DCX-positive cells was dramatically elevated by 2.2-fold to 51,586 ± 15,426/mm^3 (p=0.003, Figure 2.6 I and J). These results indicated that severe, but not mild or moderate injury, increases immature neuron number within the HDG at 2 weeks post-trauma.

TBI severity affects the generation of new mature neurons

To examine the number of post-injury newly born mature neurons after different severities of TBI, BrdU staining was combined with NeuN, a specific cell type marker for mature neurons, to detect adult-born mature neurons post-trauma (Figure 2.7 A). In the sham HDG, BrdU-positive cells mainly located in the GCL, where newly generated granule neurons are supposed to be (Figure 2.7 B). Mild

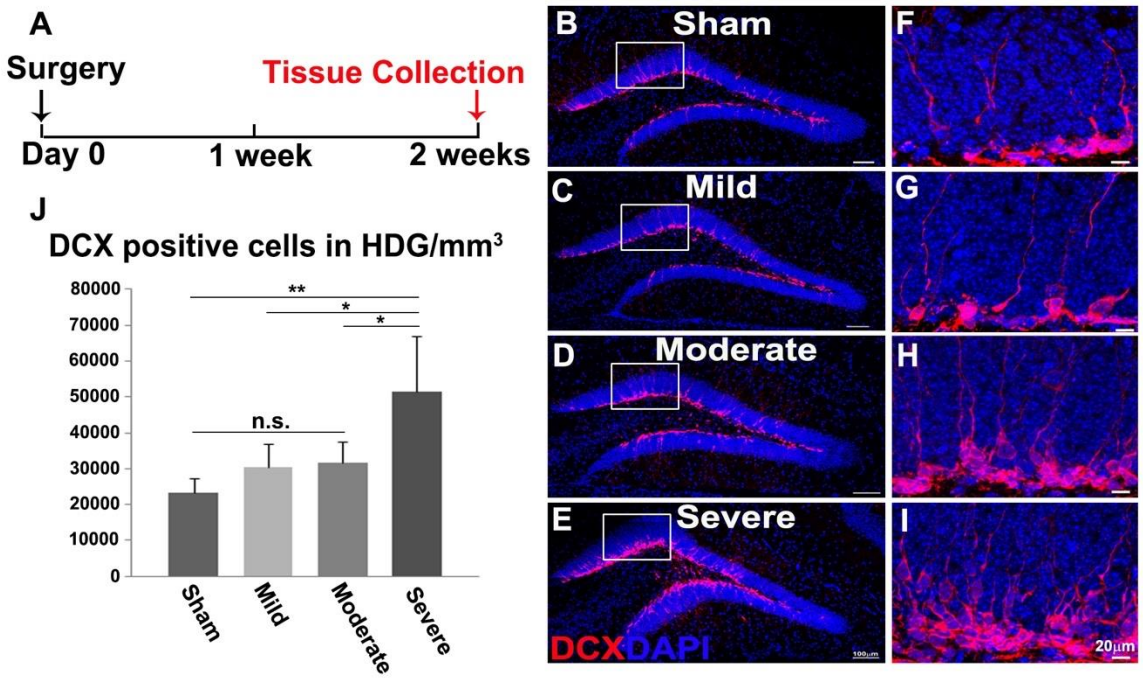


Figure 2.6: TBI severity affects immature neuron number.

(A) Schematic shows the experimental strategy. (B-I) Immunostaining against doublecortin (DCX) (red) to identify immature neurons in the hippocampal dentate gyrus (HDG) in sham (B, F), mild (C, G), moderate (D, H), and severe TBI (E, I) mice. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows the HDG structure. (F-I) Enlarged images of B-E (indicated in white boxes) to show DCX-positive immature neurons in the granule cell layer (GCL) in higher magnification. (J) Quantification of DCX-positive immature neurons after sham surgery and different TBI severities. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. not significant, n=4 for sham and moderate TBI groups, n=5 for mild TBI group, and n=7 for severe TBI group).

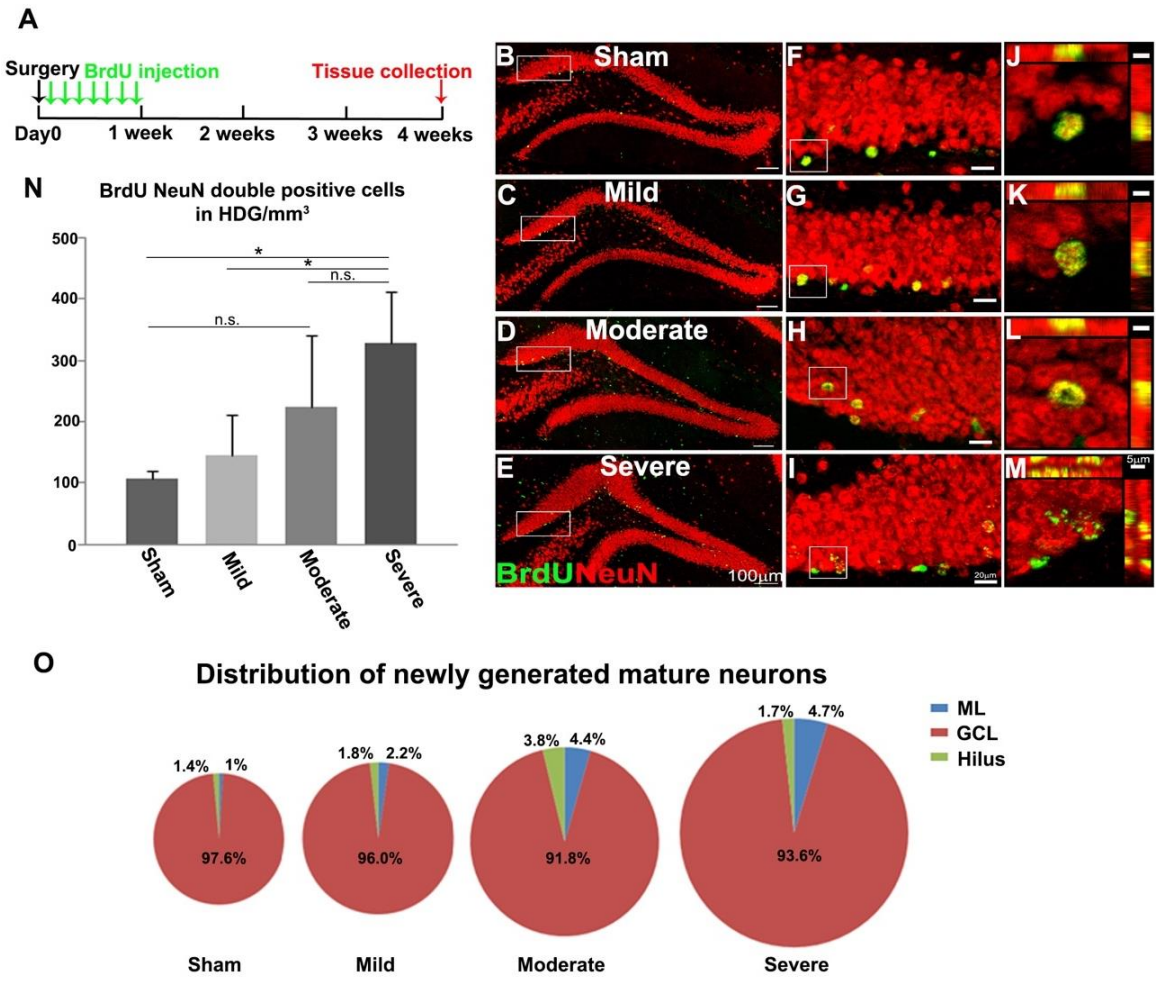


Figure 2.7: TBI severity affects mature neuron generation.

(A) Schematic shows experimental strategy. (B-M) Double immunostaining with 5-bromo-2'-deoxyuridine (BrdU) (green) and Neuronal Nuclei (NeuN) (red) to identify newly generated mature neurons in the hippocampal dentate gyrus (HDG) of sham (B, F, J), mild (C, G, K), moderate (D, H, L), and severe TBI (E, I, M) animals. (F-I) Enlarged images from B-E (indicated in white boxes) to show BrdU and NeuN double-positive cells in the granular cell layer (GCL). (J-M) 3-dimensional reconstruction of newly generated mature neurons in F-I (indicated in white boxes) to show BrdU and NeuN co-staining. (N) Quantification of BrdU and NeuN double-positive cells in HDG after sham surgery and different TBI

severities. (O) Percentage of BrdU and NeuN double-positive cells in the individual HDG subregions, molecular layer (ML), GCL, and hilus, after sham surgery and different TBI severities. Pie chart sizes indicate the ratio of total BrdU and NeuN double-positive cell number in different groups. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. not significant, n=3 for sham group, n=4 for mild and moderate TBI groups, and n=5 for severe TBI group)

TBI animals showed a similar pattern of BrdU-positive cells localization, mostly in the GCL (Figure 2.7 C). While in the HDG of moderately and severely injured animals, a large ratio of BrdU-positive cells also distributed in the ML and hilus (Figure 2.7 D and E), conferring robust gliogenesis. I further quantified BrdU and NeuN double-positive cells in different groups (Figure 2.7 F-N). In sham animals, a baseline of 108 ± 11/mm^3 new neurons were generated in the HDG (Figure 2.7 F, J, and N). The number was not significantly increased in mild TBI (145 ± 66/mm^3, p=0.935, Figure 2.7 G, K, and N) or moderate TBI (223 ± 118/mm^3, p=0.316, Figure 2.7 H, L, and N). However, severe TBI dramatically promoted newly produced neurons by 3-fold (329 ± 84/mm^3, p=0.016, Figure 2.7 I, M and N). Regardless of injury severities, the vast majority of newly generated mature neurons (more than 91%) located in the GCL with few in the ML and hilus, and there was no significant difference for the distribution among four groups (Figure 2.7 O). Taken together, the generation of adult-born mature neurons was promoted only in animals with severe, but not mild or moderate TBI. This conclusion agrees with my colleagues' previous report that demonstrated moderate CCI-induced injury does not increase neurogenesis (Gao and Chen, 2013).

TBI severity affects gliogenesis

Glial response after TBI is another source of cell proliferation besides NSC proliferation. As shown in Figure 2.3, the number of proliferated cells in the HDG increased as the severity of injury increased. Furthermore, the localization of the

proliferated cells was different following different severities of TBI. In sham or mildly injured mice, the proliferated cells primarily resided in the SGZ. In contrast, after moderate or severe TBI, proliferated cells widely dispersed to other regions in the HDG. Since the location of cells can partially indicate their identities, I first divided surviving proliferated cells into different subregions of the HDG (Figure 2.3 G-J). Compared with sham animals, mildly injured mice showed a similar pattern of BrdU-positive cells distribution in the hilus, the granule cell layer (GCL), and the molecular layer (ML). In moderate and severe TBI animals, the distribution of BrdU-positive cells shifted slightly away from the GCL (43.5% in sham and 41.1% in mild TBI compared with 31.5% in moderate and 33% in severe TBI animals in the GCL, Figure 2.3 G). When each subregion was separately examined, mild TBI did not change the number of BrdU-positive cells in any of the subregions, while moderate and severe injury increased the cell number in both the GCL and the ML, but not in the hilus. Specifically, moderate TBI resulted in a 4.6-fold and a 2.8-fold promotion of BrdU-positive cell number in the ML and the GCL, respectively. Severe TBI further elevated the number to a 5.5-fold in the ML and a 4.2-fold in the GCL (Figure 2.3 H-J). Since the majority of cells in the ML are glia, and neurons mainly reside in the GCL, adult-born mature granule neurons only account for a small part of those BrdU labeled cells. The rest of the BrdU-positive cells are likely reactive glia.

To further confirm glial cell identities, I double-stained BrdU-positive cells with different types of glial cell markers: glial fibrillary acid proteins (GFAP) for astrocytes (Figure 2.8 A-C), NG2 for oligodendrocyte precursors (Figure 2.8 D-

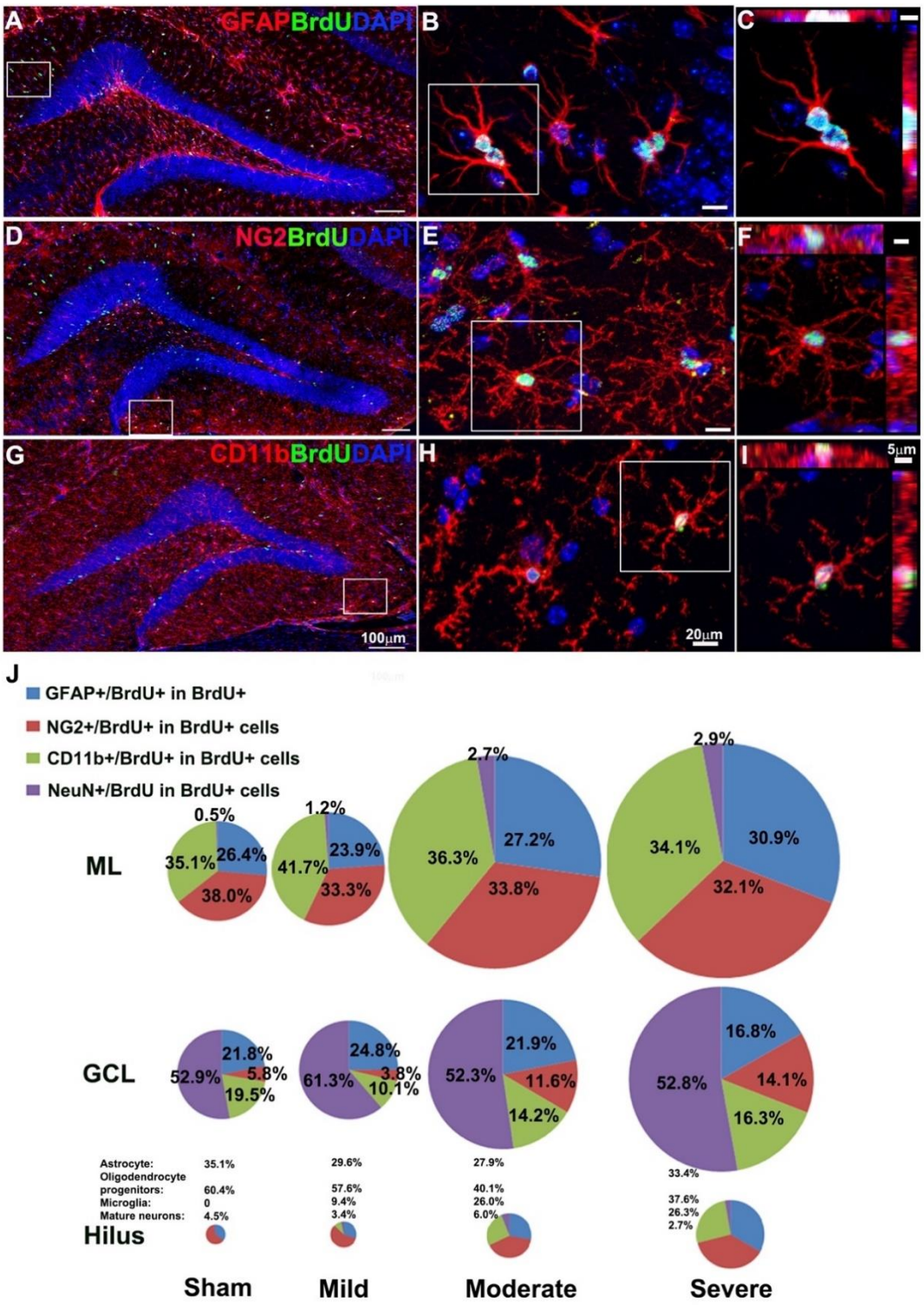


Figure 2.8: Gliogenesis accounts for the rest of surviving proliferated cells. Brain tissues were collected as the procedure shown in Figure 2.3; tissues from moderate traumatic brain injury (TBI) mice shown as an example. (A-C) Double

immunostaining against 5-bromo-2’-deoxyuridine (BrdU, green) and glial fibrillary acid proteins (GFAP, red) to identify astrocytes in proliferated cell population in the ipsilateral side of the injured brain. (D-F) Double immunostaining against BrdU (green) and NG2 (red) to identify the oligodendrocyte lineage in proliferated cell population in the ipsilateral side of the injured brain. (G-I) Double immunostaining against BrdU (green) and CD11b (red) to identify microglia in proliferated cell population in the ipsilateral side of the injured brain. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows the hippocampal dentate gyrus (HDG) structure. (B, E, H) Enlarged images of A, D, G (indicated in white boxes) to show gliogenesis in higher magnification. (C, F, I) 3-dimensional reconstruction of proliferated glial cells in B, E, H (indicated in white boxes) to confirm their cell types. (J) Percentage of different cell types in BrdU-positive surviving proliferated cell population in individual HDG subregions, molecular layer (ML), granule cell layer (GCL), and hilus, in sham and different TBI severities. Pie chart sizes indicate ratios of total BrdU-positive cell number in different groups. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, n.s. not significant, n=3 for sham group, n=4 for mild and moderate TBI groups, and n=5 for severe TBI group)

F), or CD11b for microglia (Figure 2.8 G-I). I quantified the proportion of each cell type in the BrdU-positive cell population in each subregion (Figure 2.8 J). Despite no significant alteration was detected in the cellular compositions in any of the subregions, some subtle changes were observed. In the ML, the composition of each cell type did not significantly change among different groups (Figure 2.8 J, top panel). In the GCL, the generation of mature neurons accounted for a similar ratio in the BrdU-positive cell population of all groups (52.9% in sham, 61.3% in mild, 52.3% in moderate, and 52.8% in severe TBI animals, Figure 2.8 J, middle panel), despite the absolute number of newly generated neurons increased in severe injured mice. Whereas, an increase in proliferated NG2-positive cell ratio was observed in moderate and severe injured mice (5.8% in sham, 3.8% in mild, 11.6% in moderate, and 14.1% in severe TBI, Figure 2.8 J, middle panel). On the contrary, an interesting elevation of proliferated CD11b-positive microglia ratio instead was noticed in the hilus (0 in sham, 9.4% in mild, 26.0% in moderate and 26.3% in severe TBI, Figure 2.8 J, bottom panel).

Anatomical integration of post-injury born neurons

Although I demonstrated that, in terms of number of neurons born after injury, neurogenesis level is affected by injury severities, it is not clearly studied if they function as mature neurons comparable to their counterparts born and developed in a non-injured environment or not. I wondered whether they form proper connections with appropriate presynaptic neurons or form aberrant connections and disrupt information flow. Thus, I assessed if the injury environment alters the

anatomical integration pattern of post-injury born neurons, which might affect their functions.

I used a pseudotyped rabies virus based dual-virus retrograde monosynaptic tracing system to identify the pre-synaptic neurons innervating post-injury born neurons (Figure 2.9 A). I first used a retrovirus-based helper virus to specifically infect proliferating cell, including proliferating NSCs. Once an infected cell becomes a neuron and express synapsin, a histone-tagged GFP, TVA receptor, and rabies glycoprotein are expressed under the synapsin promoter. This strategy ensures that only post-injury born neurons express the elements and are labeled by histone-GFP (Figure 2.9 B). Then EnvA pseudotyped engineered rabies virus was introduced, and would only infect cells expressing TVA receptors, which would be pre-infected post-injury born neurons. With the rabies glycoprotein expressed by the helper virus, the rabies virus conducts self-package and retrogradely infects pre-synaptic neurons. Within the infected pre-synaptic neurons, rabies virus expresses DsRed by its engineered genome and would not further infect second order pre-synaptic neurons because of its glycoprotein gene removal. Together, any post-injury born neurons infected by two viruses would be labeled by histone-GFP and DsRed, while their pre-synaptic neurons would only be labeled by DsRed (Figure 2.9 C) (Callaway and Luo, 2015, Vivar et al., 2012). By this method, I was able to evaluate connections made by post-injury born neurons, which might indicate their functions.

At 4 weeks after injury, I was able to detect post-injury born neurons labeled by hGFP and DsRed in the injured GCL (Figure 2.10). I have observed

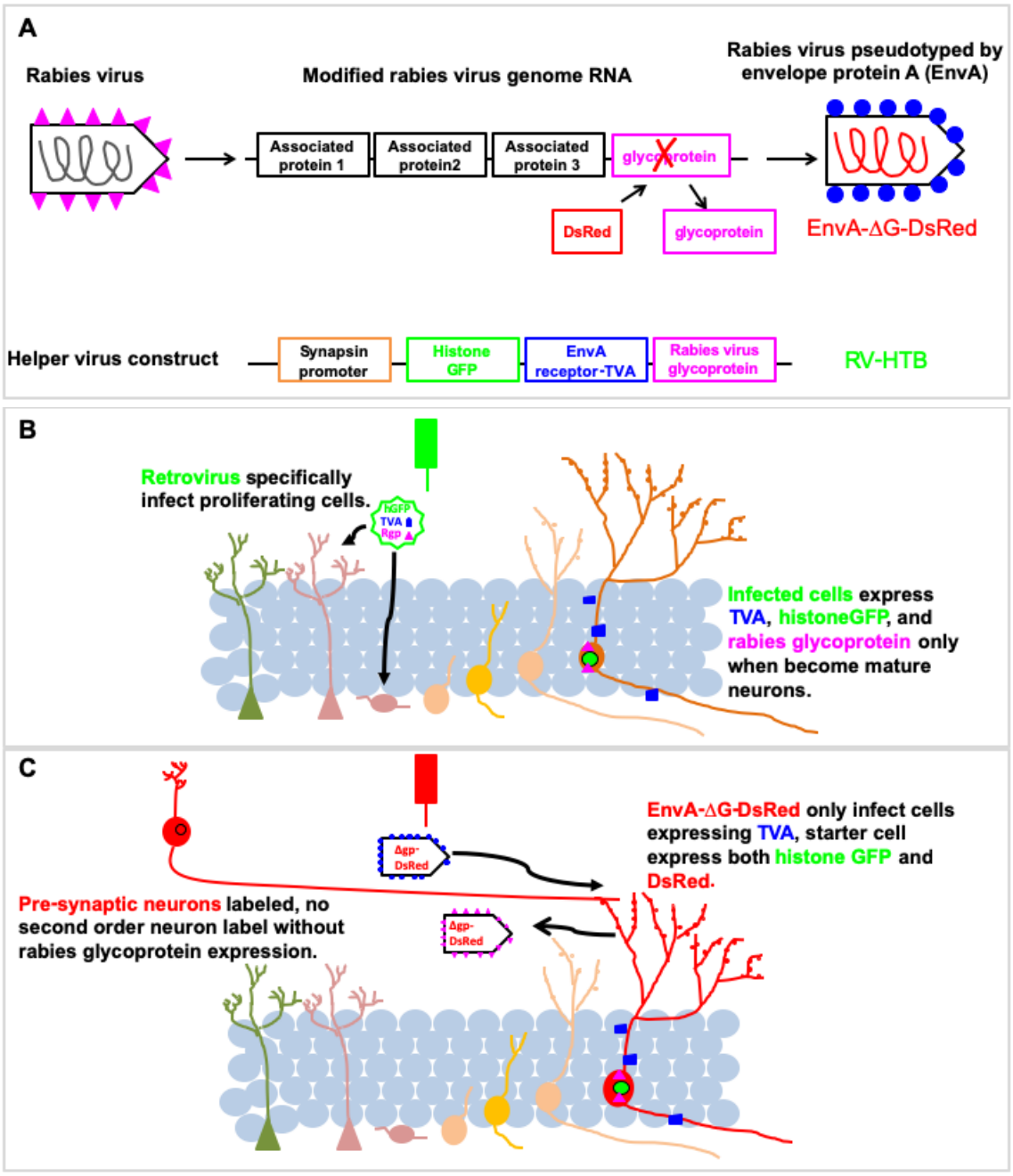


Figure 2.9: Dual-virus pseudorabies virus system of monosynaptic retrograde tracing is used to identify pre-synaptic neurons innervating post-injury born neurons.

(A) A schematic illustrates the dual-virus pseudorabies virus mediated monosynaptic retrograde tracing system. The RNA genome of rabies virus was

engineered by deleting its glycoprotein gene and replacing it with the DsRed gene. Then the engineered rabies virus genome was pseudotyped with envelop protein A (EnvA) of avian sarcoma and leukosis virus (ASLV) and named EnvA-ΔG-DsRed. EnvA drives virus infection specifically on cells expressing tumor virus A receptor (TVA). Thus, the pseudorabies virus was used together with a helper virus, which express TVA, histone tagged green fluorescent protein (hGFP), and rabies virus glycoprotein (Rgp) under a synapsin promoter. The helper virus was packaged in a retrovirus form and named it RV-HTB. Modified from Figure 1 C (Wickersham et al., 2007). (B) To trace pre-synaptic innervation of post-injury born neurons, RV-HTB was intracranially injected into the dentate granule cell layer after TBI. Retrovirus specifically infects proliferating cells, which include proliferating NSCs after TBI. Once the proliferated cells differentiate and become mature neurons, they express TVA on cell surface, Rgp, and hGFP in nuclei. (C) A week before assessing the innervation, EnvA-ΔG-DsRed was intracranially injected into the dentate granule cell layer at the same coordinates. EnvA-ΔG-DsRed only infects cells expressing TVA, which are post-injury born neurons that have been pre-infected by RV-HTB. These double infected cells are termed starter cells. Then the pseudorabies virus gets packaged by Rgp expressed in the starter cell, which then retrograde label its pre-synaptic neurons.

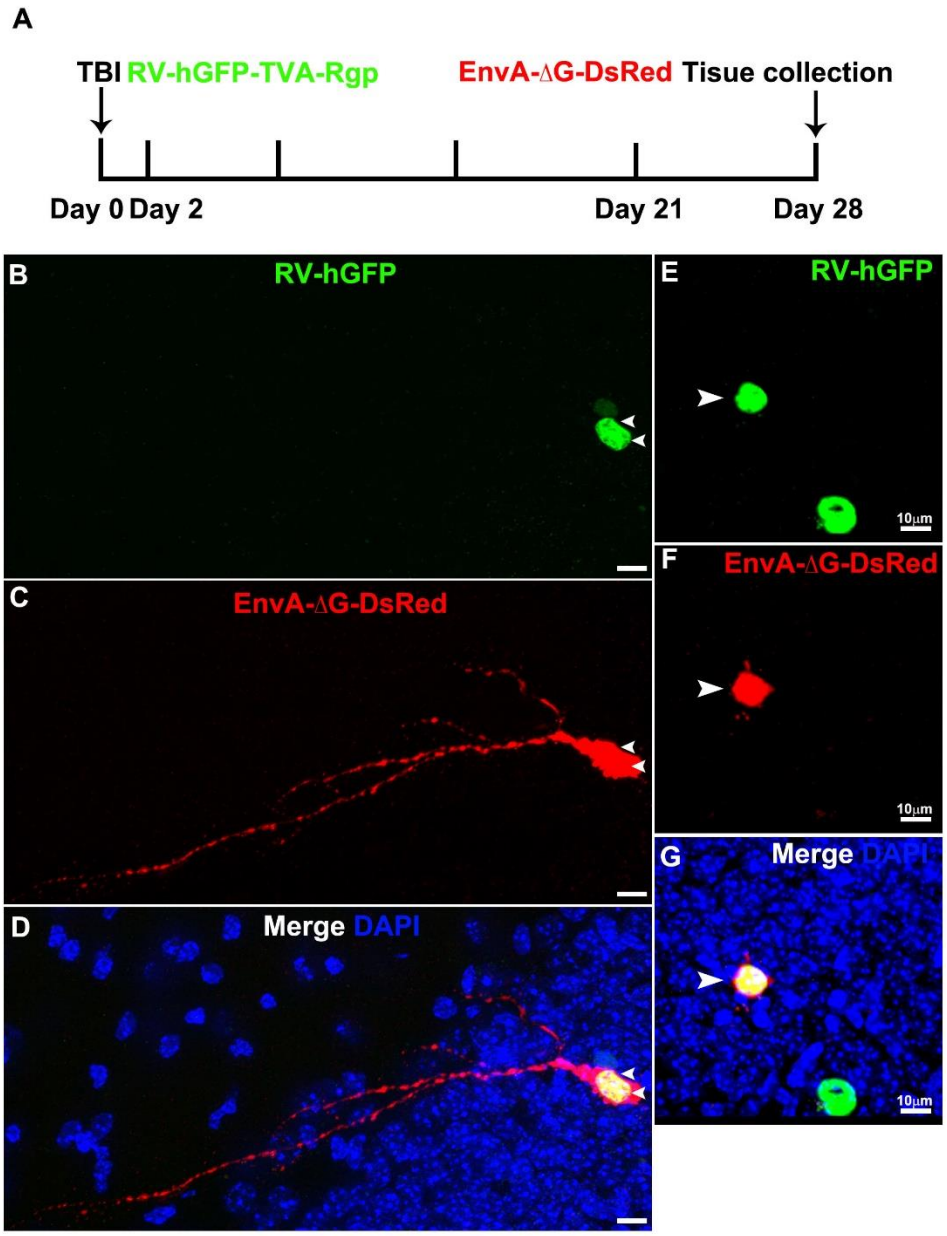


Figure 2.10: Post-injury born neurons infected by dual viruses.

(A) A schematic shows the experimental design. (B-G) Immunostaining with GFP (green) and DsRed (red) to identify post-injury born neurons as starter cells (indicated by white arrowhead) in the granule cell layer. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows the hippocampal dentate granule cell layer structure.

pre-synaptic neurons locally in the ipsilateral granule cell layer, dentate molecular layer, hilus, and CA1 regions, matching connections locally in the hippocampus (Figure 2.11), and distally in the contralateral perirhinal cortex (PRH), ipsilateral retrosplenial granular cortex (RSG), and ipsilateral medial habenular nucleus (MHb, Figure 2.12). These results proved that post-injury born neurons are able to integrate into neural networks. The connections suggest that post-injury born neurons' activity is possibly regulated under local feedback circuit, and they are highly likely participating in memory functions and integrating sensory, spatial, and emotional information.

Functional integration of post-injury born neurons

With the dual-virus system, I illustrated the anatomical integration of post-injury born neurons. I further wondered if they are able to exert neuronal activity to support functional outcomes. Previous studies have widely used expression of immediate early gene, c-fos, as a marker to visualize functional integration of neurons (Kee et al., 2007, Garner et al., 2012, Ramirez et al., 2013). Thus, in the current study, I similarly used the expression of c-fos as an indicator of functional integration of post-injury born neurons. I labeled cells born in the first 2 weeks after injury by BrdU incorporation and collected brain tissues after 6 weeks of TBI (Figure 2.13 A). By the BrdU, NeuN, and c-fos triple labeling strategy, I evaluated functional integration of post-injury born neurons at their age of 4 weeks to 6 weeks, the time point when newborn neurons have been proved able to functionally integrate into network (Kee et al., 2007). I was not able to detect

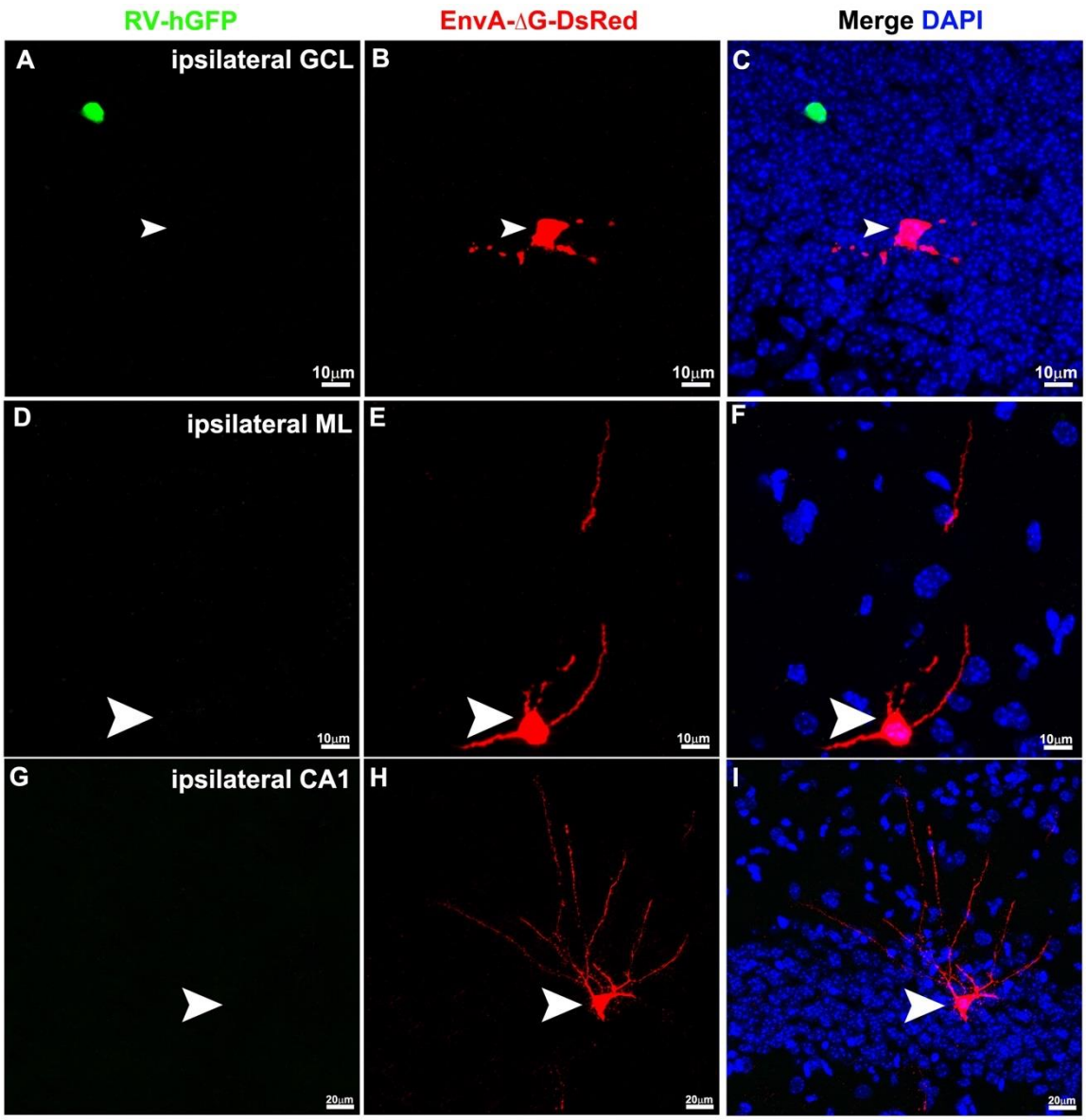


Figure 2.11: Local pre-synaptic neurons within the hippocampus innervating post-injury born neurons labeled by DsRed.

(A-I) Immunostaining with GFP (green) and DsRed (red) to identify pre-synaptic neurons (indicated by white arrowheads) in the hippocampal granule cell layer (A-C), hippocampal dentate molecular layer (D-F), and hippocampal CA1 (G-I) that innervates post-injury born neurons. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows the hippocampal dentate granule cell layer structure.

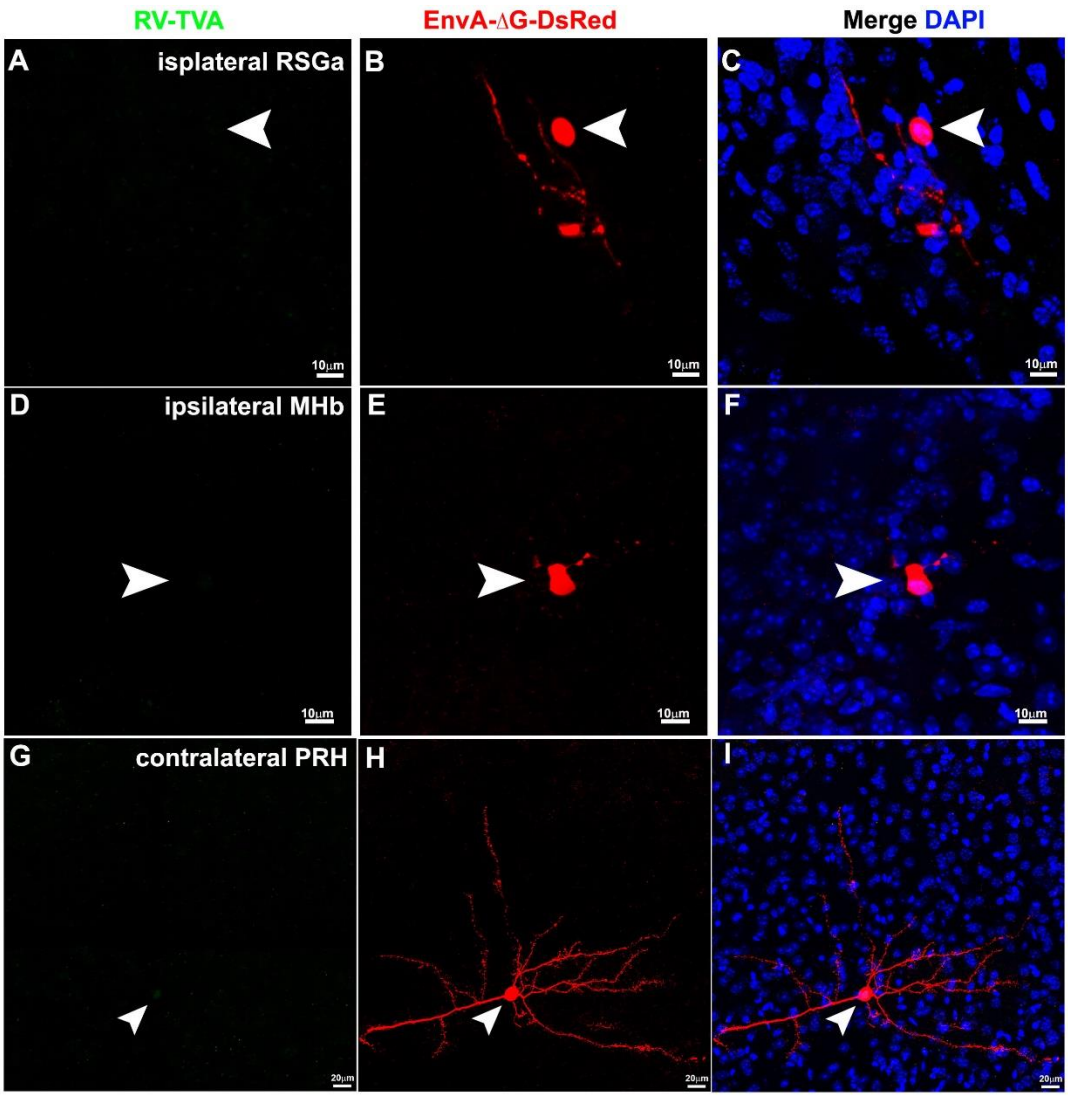


Figure 2.12: Distal pre-synaptic neurons in cortical and subcortical regions innervating post-injury born neurons labeled by DsRed.

(A-I) Immunostaining with GFP (green) and DsRed (red) to identify pre-synaptic neurons (indicated by white arrowheads) in the retrosplenial granular cortex (A-C), medial habenular nucleus (D-F), and perirhinal cortex (G-I) that innervates post-injury born neurons. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows the brain structure. MHb: medial habenular nucleus; PRH: perirhinal cortex; RSGa: retrosplenial cortex a).

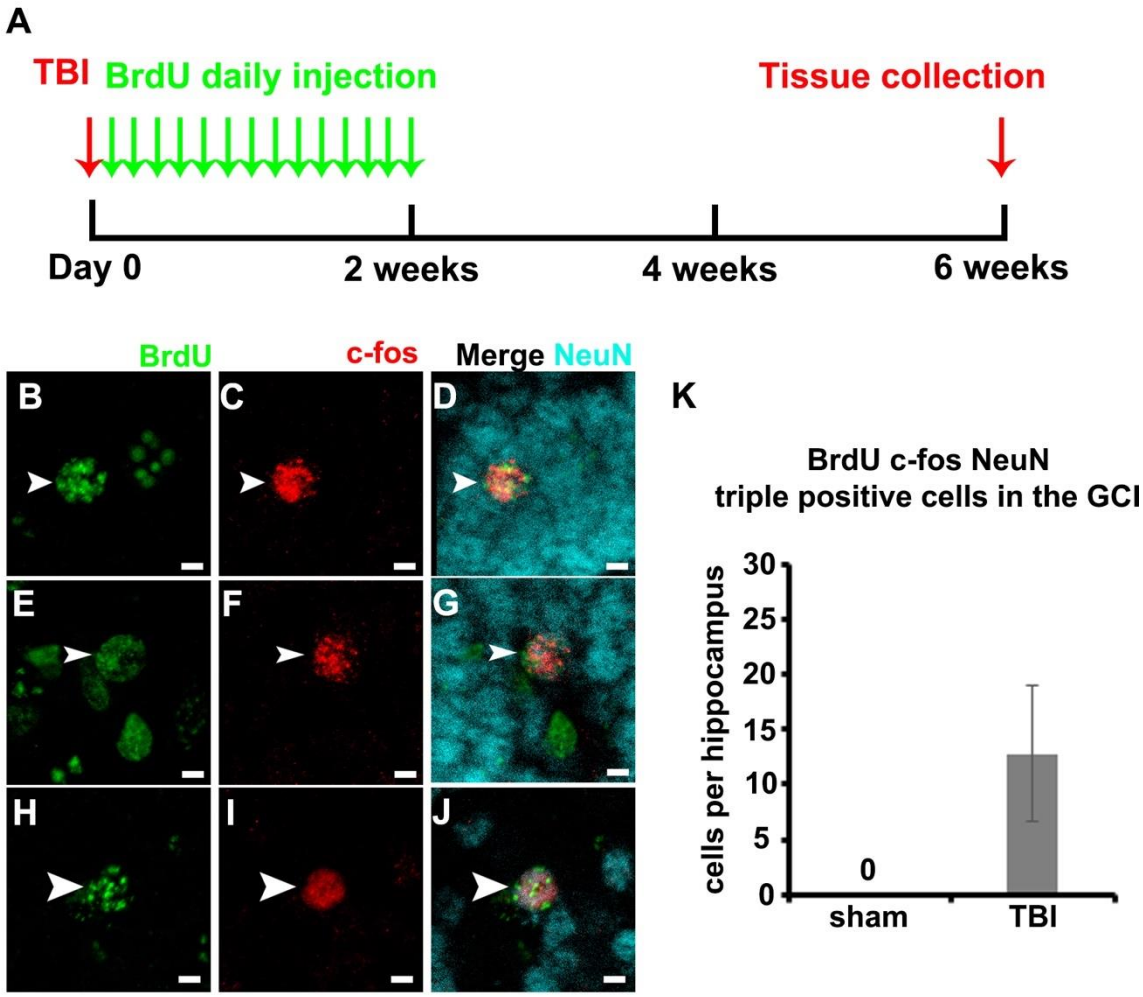


Figure 2.13: Functional recruitment of post-injury born neurons in a standard housing environment.

(A) A schematic shows the experimental design. (B-J) Immunostaining with BrdU (green), c-fos (red), and NeuN (cyan) to identify neuronal activity in post-injury born neurons (indicated by white arrowheads, all from TBI animals). Scale bar indicates 5 μm. (K) Quantification of active post-injury born neurons in sham and TBI animals (n=4 for each group).

triple positive cells in sham animals (0 triple positive cells in 1,809 ± 893 BrdU and NeuN double positive post-surgery born neurons). On rare occasions, I was able to observe active post-injury born neurons in TBI animals (13 ± 6 triple positive cells in 5,732 ± 1,964 BrdU and NeuN double positive post-injury born neurons) (Figure 2.13 B-K). Although rare, this quantification suggested that post-injury born neurons were able to exert functional activities in a standard housing environment.

Furthermore, I asked if the post-injury born neurons are able to respond to behavior task. To answer the question, I challenged mice with standard Morris water maze (MWM) test at 7 weeks after TBI and sacrificed mice 45 min after MWM probe test (Figure 2.14 A). After MWM test, I was able to detect triple positive cells in both sham and TBI animals (Figure 2.14 E-G), and there was a slight but not significant increase of number of triple positive cells in TBI than sham cohorts (13 ± 7 triple positive cells in sham compared to 20 ± 7 in TBI, p=0.13, Figure 2.14 H). Further studies are needed to validate if TBI promotes functional recruitment of newborn neurons or not.

Discussion

NSCs support adult neurogenesis in a lifetime and resemble the regenerative potential of the adult brain. NSC proliferation in the hippocampus after TBI sheds light on the possibility of injury repair by endogenous neurogenesis. Whereas, whether the NSC proliferation supports neurogenesis after injury is argued. Current contradictory studies were conducted in different injury models, mostly in

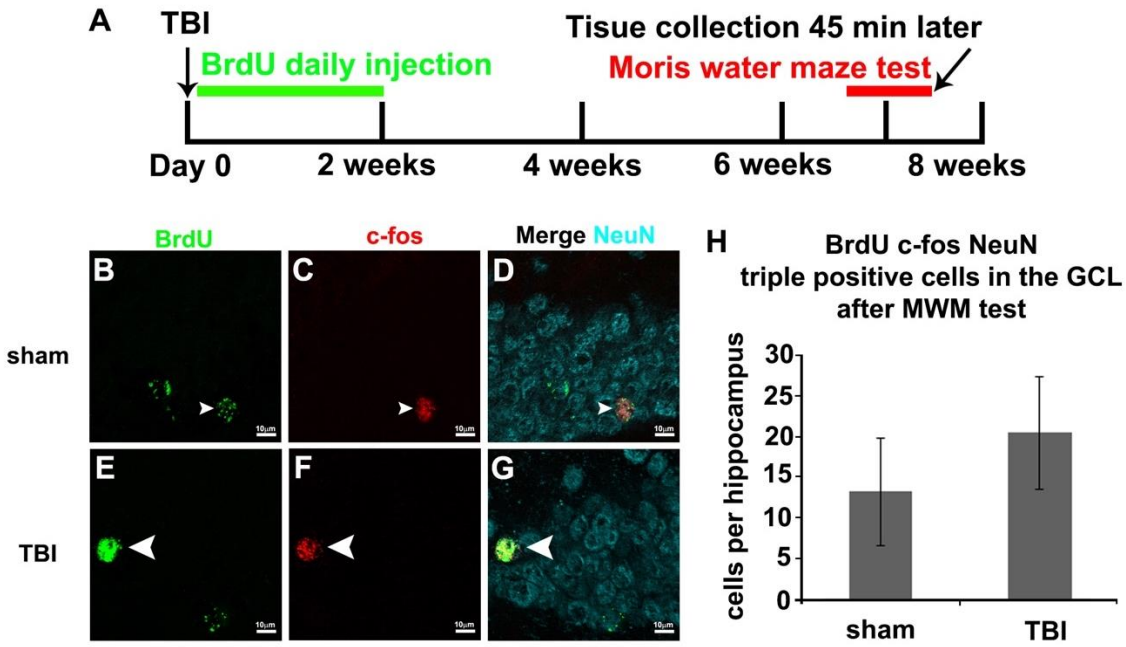


Figure 2.14: Functional recruitment of post-injury born neurons upon Morris water maze test.

A) A schematic shows the experimental design. (B-G) Immunostaining with BrdU (green), c-fos (red), and NeuN (cyan) to identify neuronal activity in post-injury born neurons (indicated by white arrowheads) in sham (B-D) and TBI (E-G) animals. (H) Quantification of active post-injury born neurons in sham and TBI animals after Morris water maze test (n=5 for each group).

rodents. Comparing reported works, CCI injury induced in adult mice (Gao and Chen, 2013, Rola et al., 2006) and cortical contusion injury induced in adult rats (Braun et al., 2002) both recapture focal injury characteristics in human TBI. Whereas impact acceleration model performed in adult rats (Bye et al., 2011) mainly mimics diffused axonal injury in patients, and lateral FPI injury induced in adult rats (Chirumamilla et al., 2002, Sun et al., 2005, Sun et al., 2007) exhibits mixed focal and diffuse injury in the clinical setting (Morales et al., 2005). Diffuse injury, i.e. impact acceleration and FPI, tends to affect a larger brain area than focal injury, i.e. cortical contusion model and CCI, so diffuse models generally produce injury with greater severity regarding the whole brain network. Overall, neural activity, neurotrophic factors, and metabolism level in the hippocampus may all be altered by various injury models and resulting in injury severity differences. Since all the signals above are essential for adult neurogenesis regulation (Faigle and Song, 2013), injury severity is considered as one of the most crucial variations in previous discrepancies. Besides injury severity, the time when neurogenesis was examined is also critical, as TBI pathogenesis and adult neurogenesis are both dynamic processes. Current studies that evaluated neurogenesis at as early as 2 days after injury to as late as 10 weeks after injury might reflect different single points on the dynamic curve. Here, I assessed neurogenesis at three crucial stages after different severities of TBI in a consistent system, to attain a better understanding of how NSCs respond to trauma.

By combining BrdU labeling and immunostaining of specific cell type markers, I evaluated the effects of injury severity on NSC proliferation, immature neuron number, and maturation of adult-born neurons (Figure 2.15). In my study, I observed that mild TBI hardly causes alteration of NSC proliferation, immature neuron number, or generation of mature neurons, implying low NSC or glial responses. Previously, my colleague reported that mild TBI causes limited cell death in the cortex but not in the HDG (Gao and Chen, 2011), and this may explain why mild TBI does not trigger cell proliferation and following processes.

Moderate TBI results in increased NSC proliferation without dramatic change in immature neuron number or mature neuron production. My colleagues have reported that immature neurons are the most vulnerable cells in the hippocampus, leading to a dramatic decrease of the immature neuron number in the hippocampus at 24 h after injury (Gao et al., 2008). Although moderate injury promotes NSC proliferation, it only makes up for the loss of immature neurons in the early acute phase. The slight but not significant increase of immature neuron number is likely compensating effect.

In contrast, severe CCI injury induced a much more severe cortical tissue lesion and increased cell death, primarily mature neuron death, in the dentate gyrus. Severe CCI significantly promoted NSC proliferation and mature neuron generation compared with moderate TBI, corresponding to the drastic mature neuron death when injury severity increased. This result indicates that NSCs can sense the environmental changes and respond to different intensities of stimuli.

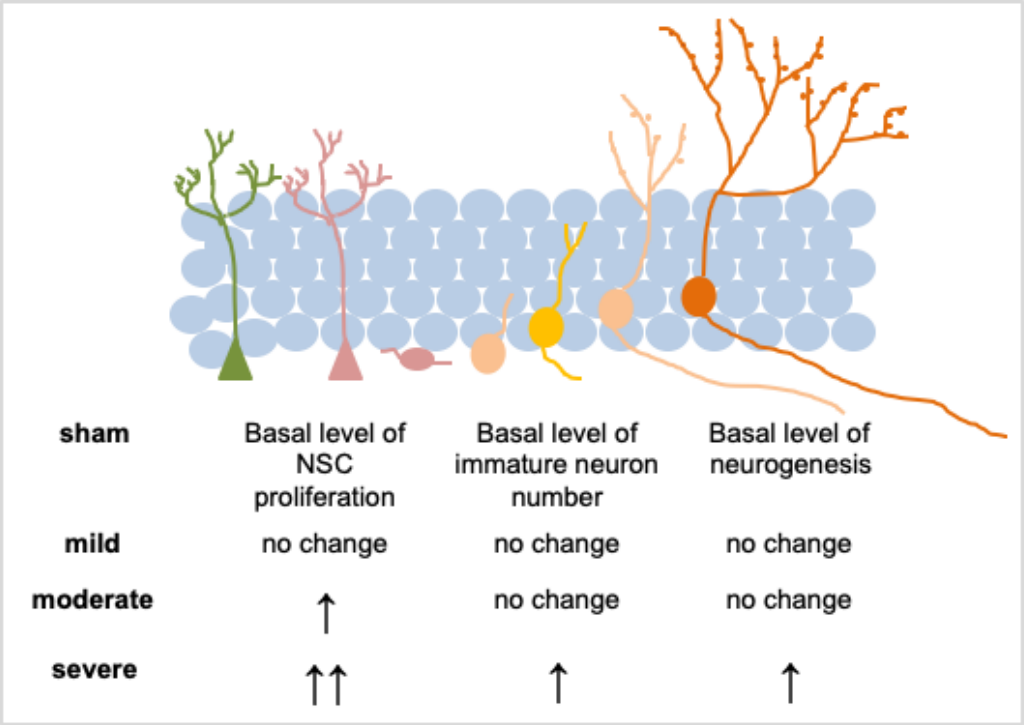


Figure 2.15: Summary of post-traumatic neurogenesis in a severity-dependent manner.

A schematic summarizes the severity-dependent post-traumatic neurogenesis. Sham animals have a baseline of NSC proliferation, immature neuron number, and mature neuron generation. Mild TBI does not change any of the stages. Moderate TBI increases NSC proliferation, which makes up for the immature neuron loss. Thus, immature neuron number returns to a comparable level of sham animals, and generation of mature neurons is not changed. Severe TBI further increases NSC proliferation, increases immature neurons, and increases the generation of mature neurons.

Subtle changes in gliogenesis were also detected in different subregions of the HDG. Cellular composition was comparable among all groups in the ML. Moderate and severe CCI caused an increased percentage of proliferated NG2 cells in the GCL. The proliferated microglia ratio was elevated in all TBI groups instead in the hilus. It is interesting to observe these trends, since glial cells play crucial roles in regulating neuronal activity and adult neurogenesis. Traditionally considered solely as oligodendrocyte precursors, NG2 cells have currently been shown to have population heterogeneity. Some subgroups of NG2 cells can respond to neuronal stimulation in the adult hippocampus by elevating intracellular calcium. They are considered as modulators of synaptic activity (Trotter et al., 2010). NG2 cells in the GCL after injury predominantly located close to the inner or outer border of the GCL, thus indicating their potential involvement in modulating neuronal activity in spared or newly formed neural circuitry post injury. Microglia have been reported to play bidirectional roles in adult neurogenesis. Classic activation of microglia was reported to inhibit neurogenesis by expressing pro-inflammatory cytokines, such as IL-6 and TNF α, while with alternative activation, microglia release anti-inflammatory cytokines, i.e. IL-4, IGF-1 and TGF β, and promote neurogenesis (Belarbi and Rosi, 2013). In my case, microglia subtype identification was not performed. Although the elevated microglia proportion in the hilus is likely one of the mechanisms regulating neurogenesis, no clear clue refers to their supportive or detrimental involvements to post-traumatic neurogenesis.

Taken together, my results demonstrate that TBI severity affects post-traumatic neurogenesis. Mild TBI does not affect post-traumatic neurogenesis. Moderate TBI promotes post-traumatic NSC proliferation without increasing neurogenesis. Severe TBI enhances post-traumatic NSC proliferation, immature neuron number, and mature neuron generation (Figure 2.15). By illustrating the phenomenon above, I may partially explain the existing dispute among different groups regarding post-traumatic neurogenesis. Moreover, this effect on post-traumatic neurogenesis indicates that in the adult brain, NSCs have the potential to compensate for cell loss following injury. By further studying the mechanism that regulates this inner neuroplasticity, I might provide insights into a potential therapeutic application of endogenous NSCs against cell loss post-trauma.

Previous studies on individual post-injury born neurons have shown that they have both similarities and differences compared to their counterparts in sham animals regarding their morphologies, electrophysiological properties, and their location. Neurons born after TBI showed similar membrane potential, excitability, spine density, and response to perforant pathway stimuli compared to their counterparts in sham animals (Villasana et al., 2015). However, they exhibited altered dendritic arborization by branching closer to soma, growing more apical dendrites, and/or spreading branches to a larger angle (Villasana et al., 2015). Additionally, adult-born neurons normally migrate slightly from SGZ to inner GCL (Zhao et al., 2006), but post-injury born neurons showed ectopic migration and misplaced in the outer GCL or even in the ML (Villasana et al., 2015, Ibrahim et al., 2016). Together, these observations indicate the possibility

of post-injury born neurons forming synapses with neurons that they might not connect in a non-injured environment, which might disrupt network and perform maladaptive functions. Indeed, aberrant neurogenesis has been widely proposed as a mechanism for epilepsy (Jessberger and Parent, 2015). Thus, it is critical to investigate the anatomical integration pattern of post-injury born neurons to illustrate their beneficial and/or maladaptive functions.

In normal conditions, developmentally-born granule neurons are classically studied and known to primarily receive glutamatergic signals from the perforant path of entorhinal cortex (EC) layer II neurons. Additionally, they receive cholinergic projections from the medial septal nucleus (MS) and the nucleus of the diagonal band of Broca (NDB), and projection from the supramammilary nuclei, which are likely excitatory via glutamate. Locally, their dendrites also form synapses with Mossy cells, basket cells, and other hilar interneurons. They relay signals to CA3 pyramidal neurons through the mossy fiber pathway (Andersen and Oxford University Press., 2007). Separately, adult-born neurons have been specifically studied with the dual-virus system and have shown some similar but also unique connections compared to the developmental counterparts. They successively receive the classic inputs from local mossy cells and interneurons, followed by distal inputs from LEC, MS and NDB, but also uniquely receive inputs from local mature granule neurons, CA3 pyramidal neurons, subiculum, and distal perirhinal cortex (Vivar et al., 2012, Deshpande et al., 2013). The temporal shift of input connections is considered critical for regulating adult-born granule neuron survival and functional maturation.

In my study, I observed the innervation of post-injury born neurons from PRH, local granule neurons, and hilar neurons, consistent with their integration pattern in the non-injured environment, indicating normal functions they may serve. Novel inputs from the medial habenular nuclei (MHb) and retrosplenial granular cortex (RSG) were also discovered in the injured animals. However, studies of pre-synaptic tracing on adult-born granule neurons are limited, it is too early to conclude if these novel inputs represent undiscovered connections specific to adult-born granule neurons or misconnections made by post-injury born neurons. Additionally, MHb is known to participate in anxiety and fear memory formation (Viswanath et al., 2013), while the RSG is involved in episodic memory, navigation, and learning (Vann et al., 2009). These novel connections are highly consistent with the proposed roles of post-injury born neurons in cognitive functions, especially learning and memory, supporting the beneficial roles of post-traumatic neurogenesis. Further investigations on anatomical integration of post-injury born neurons at more and longer time points after TBI would help understand the temporal shift of signals that post-injury born neurons receive, provide more detailed evaluations on the anatomical integration of post-injury born neurons. This will shed light on the normal and/or maladaptive functions of post-injury born neurons.

Functionally, adult-born neurons were studied for their integration by their expression of IEGs, such as c-fos, Arc, Zif268, and Homer1A. Adult-born neurons were able to express IEGs in animals housed in home cage and respond to environment exploration behavior, MWM test, and drugs-induced

seizures (Carlen et al., 2002, Jessberger and Kempermann, 2003, Ramirez-Amaya et al., 2006). Compared to their developmentally born counterparts, using c-fos as an indication of functional integration, adult-born neurons were proved to be preferentially recruited into spatial memory network by the age of 4 weeks or more (Kee et al., 2007). Besides spatial learning and memory in MWM test, adult-born neurons' roles were studied in contextual fear memory and spatial pattern separation by fear conditioning test and the touchscreen system, respectively (Nakashiba et al., 2012, Vivar et al., 2012, Gu et al., 2012). By ablating different populations of adult-born neurons at specific ages, non-reversibly silencing subgroups of adult-born neurons by tetanus toxin, or optogenetically silencing adult-born neurons, they were learned to be specifically required at the age of 3 to 4 weeks for spatial memory retrieval, long-term memory retention, and contextual discrimination (Gu et al., 2012, Nakashiba et al., 2012, Deng et al., 2009). Together, adult-born neurons are able to functionally contribute to behaviors at least after 4 weeks of their birth (Kee et al., 2007).

In current studies, I also used c-fos as a marker to investigate the functional integration of post-injury born neurons. In a home caged standard environment, I did not detect c-fos expression in newborn neurons aged 3-4 weeks in sham animals or TBI injured animals, possibly due to their early maturation stage and/or limited stimuli in standard home caged environment. While for post-injury newborn neurons aged 4-6 weeks, I detected c-fos expression in these cells in TBI injured animals but not in sham animals in home

caged standard environment, indicating possible accelerated maturation of post-injury born neurons by TBI. Moreover, I detected c-fos expression in newborn neurons aged 5-7 weeks after MWM spatial memory test in both sham and TBI animals, supporting functional integration of post-injury newborn into spatial neural networks. The number of c-fos positive post-injury born neurons observed in my studies are relatively limited, consistent with the sparse coding activity pattern of HDG granule cells (Jung and McNaughton, 1993). Noteworthily, I again observed a slight increase of post-injury newborn neurons' integration in TBI compared to sham animals, indicating potential accelerated maturation of neurons born in the injured environment. Further investigations on potential mechanisms of this phenomenon would enhance the current understanding of the functional contributions of post-injury born neurons to the neurobehavioral improvements.

Collectively, the body of my works in chapter 2 profiled the way of how TBI reshapes neurogenesis in terms of specific stages of neurogenesis affected by different injury severities, demonstrated that post-injury born neurons are able to integrate into neural network by receiving major inputs locally from neurons within hippocampus and distally from neurons in MHb, PRH, and RSG, and be functionally recruited especially upon behavior task. These conclusions serve as the foundations to investigate and test new functional improvements, especially learning and memory function restoration after TBI, by enhancing endogenous neurogenesis.

With the baseline of how TBI reshapes neurogenesis profiled, an approach to enhance post-traumatic neurogenesis is an urgent need. Although great efforts have already been devoted to clinical trials, to date, no treatment is clinically available to target post-traumatic neurogenesis. The lack of understanding molecular mechanisms governing post-traumatic neurogenesis is a major obstacle. In the following studies, I sought to investigate the molecular mechanism of TBI-enhanced NSC proliferation in hopes of providing a target for enhancing post-traumatic neurogenesis potentially by pharmacological interventions.

CHAPTER 3

IDENTIFICATION OF MTORC1 AS A MOLECULAR TARGET TO PROMOTE TBI-ENHANCED NSC PROLIFERATION

Hypotheses

1) mTORC1 is required for TBI-enhanced NSC proliferation, 2) TBI primarily promotes proliferation of radial-glial like (RGL) NSCs but not neural progenitor cells (NPCs) through activating mTORC1 signaling, and 3) mTORC1 promotes RGL NSC proliferation by priming quiescent NSCs to a *de novo* alert state.

Introduction

NSCs in the adult hippocampus have been observed to increase proliferation in response to TBI in both human patients and experimental models (Dash et al., 2001, Kernie et al., 2001, Braun et al., 2002, Chirumamilla et al., 2002, Rice et al., 2003, Ramaswamy et al., 2005, Gao et al., 2009a, Zheng et al., 2013, Sun et al., 2005). However, the spontaneous response of the adult NSCs is frequently inadequate for full compensation on the cell loss. Thus cognitive function deficits commonly persist in TBI patients (Prigatano, 1987, Cicerone et al., 2005). Although it holds great promise for utilizing the innate neurogenic machinery for injury repair, the lack of mechanistic studies on TBI-enhanced NSC proliferation impedes the development of therapeutic approaches targeting this regenerative machinery.

Hippocampal NSCs reside in a niche consists of various cell types and extracellular matrix. Multiple extracellular signals, such as parvalbumin interneuron activity, local granule neuron activity, Notch signal from progeny cells, and several others (Sierra et al., 2015, Song et al., 2012, Breunig et al., 2007, Jang et al., 2013), work together to regulate NSC activity in normal conditions. After TBI, multiple signals from the NSC niche are greatly disrupted, and it is possible that it is not a single signal alteration but rather a combination of alterations that stimulates the transient NSC proliferation increase. Instead of isolating extracellular mediators, I targeted the intracellular signaling pathway that integrate and transduce extracellular signals and modulate NSC activity post-trauma.

One of the critical intracellular signaling pathways governing cell proliferation is the mammalian target of rapamycin (mTOR) or mechanistic target of rapamycin (mTOR) pathway. The mTOR pathway is capable of orchestrating extracellular signals and regulating cell growth, proliferation, and survival through regulating protein synthesis, energy metabolism, and autophagy (Laplante and Sabatini, 2012). Dysregulated mTORC1 activity in embryonic and neonatal mice leads to imbalanced proliferation and differentiation of their NSCs (Magri et al., 2011, Hartman et al., 2013). In adult and aging rodent SVZ, mTORC1 is required for the neural progenitor pool maintenance (Paliouras et al., 2012). Decrease of mTORC1 activity in the aging mouse hippocampus contributes to NSC proliferation decline, and pharmacological activation of mTORC1 rescued the decline and increased neurogenesis (Romine et al., 2015). Taken together,

mTORC1 plays essential roles in NSC activity regulation. After trauma, mTORC1 activation was reported in the hippocampus, presumably in neurons, microglia, and astrocytes (Chen et al., 2007, Park et al., 2012). Thus, I hypothesized that mTORC1 is required for TBI-enhanced NSC proliferation. To test the hypothesis, I examined the spatial-temporal profile of mTORC1 activation in the injured hippocampus, specifically assessed mTORC1 activation in NSCs, inhibited mTORC1 activity after TBI by rapamycin and evaluated subsequent NSC proliferation. My results demonstrated that mTORC1 activation in NSCs peaks at 24 h and maintained high to 48 h post-trauma. Systemic inhibition of mTORC1 signal suppressed TBI-enhanced NSC proliferation in the hippocampus. Thus, I proved that mTORC1 is required for TBI-enhanced NSC proliferation.

NSCs in the hippocampus consists of radial glia-like cells (RGLs), the putative NSCs, and immediate progenitor cells (IPCs), the neuronal lineage committed NPCs (Bond et al., 2015). The RGL NSCs are able to self-renew and commit neuronal or astrocytic differentiation in the adult hippocampus (Bonaguidi et al., 2011, Sierra et al., 2015), while the NPCs have limited proliferative capacity and are mainly committed to neuronal differentiation (Daniel A. Berg, 2015). A previous study suggested that TBI primarily activates RGL NSC rather than NPC proliferation (Gao et al., 2009a). I hypothesized that mTORC1 activation is mediating the differential responses of RGL NSCs and NPCs. To test the hypothesis, I distinguished RGL NSC proliferation from NPC proliferation, evaluated mTORC1 activation in RGL NSCs versus NPCs, and assessed RGL NSC and NPC proliferation after TBI in the presence of mTORC1 inhibition. My

results confirmed TBI primarily activates RGL NSC but NPC proliferation, mTORC1 is activated in majority of RGL NSCs but only in a small proportion of NPCs, inhibition of mTORC1 mainly affects RGL NSC proliferation and has less of an influence on NPC proliferation. Together, I confirmed that mTORC1 activation primarily mediates RGL NSC proliferation after TBI.

NSC proliferation, especially RGL NSC proliferation, is a precisely controlled process, in which NSCs exit quiescence and enter cell cycle to proliferate, when they become active NSCs. RGL NSCs primarily remain quiescence with a small proportion activated to support neurogenesis in normal conditions. The delicate balance between quiescence and activation is regulated by multiple extrinsic signals within the NSC niche and intrinsic signals within NSCs themselves (Cheung and Rando, 2013), and the prerequisite step to activation is the exit from quiescence. Based on my data that mTORC1 is mediating TBI-enhanced RGL NSC proliferation, I further hypothesized that mTORC1 functions through promoting RGL NSC exit from quiescence and priming NSCs for possible proliferation. To test the hypothesis, I evaluated the status of individual RGL NSCs in terms of their mTORC1 activity and proliferative states in sham and TBI animals. My data showed the existence of a large proportion of RGL NSCs with mTORC1 signal activation at 24 h after injury, while the increase of RGL NSC proliferation at 48 h was not as dramatic as the mTORC1 activation. The return back to quiescence was observed in the majority of the mTORC1 activated RGL NSCs. Thus, I proposed the existence of a *de novo* state of NSCs between quiescence and activation, featured by mTORC1

activation, in which I termed them alert NSCs. I proposed that alert NSCs reversibly exit quiescence and are primed to proliferate, while they remain the capacity to return back to quiescence.

Materials and methods

Animal care

Male mice (n=83) at the age of 8-10 weeks were housed as described in chapter 2. All procedures were performed under protocols with approval from Indiana University Animal Care and Use Committee.

Controlled cortical impact

Injuries were performed as described in chapter 2. In these experiments, injury parameters were set at 3.0 m/s or 3.5 m/s, 1.0 mm, 0.1 s.

Drug administration

Rapamycin was dissolved in DMSO at a concentration of 25 mg/ml. It was further diluted to 1mg/ml in a solution of 5% PEG400/4% ethanol and 5% Tween 80 in sterile water. Rapamycin (10mg/kg, i.p.) or vehicle (5% PEG400/4% ethanol and 5% Tween 80 in sterile water) was injected at 12 h, 24 h, 36 h, and 44 h post-trauma. BrdU was given right after the final dose of rapamycin at 44 h after TBI (100 mg/kg in saline, i.p.).

Immunoblotting

To evaluate mTORC1 activity in the hippocampus after injury, mice were transcardially perfused with cold saline after CCI injury at 4 h, 24 h, 48 h, 72 h, and 1 week or after sham surgery. The ipsilateral hippocampi were freshly harvested and homogenized in ice-cold Triton lysis buffer (1% Triton, 20 mM Tris-HCL, 5 mM EGTA, 10 mM EDTA, 150 mM NaCl, and protease inhibitor cocktail [Roche, Basel, Schweiz]). Samples were centrifuged for 30 min 14000 rpm at 4 °C and subjected to protein concentration determination by a modified Lowry assay (Bio-rad, Hercules, CA). For every sample, the same amount of protein was loaded for SDS/PAGE and electrotransferred to nitrocellulose membranes at 30 V at 4 °C overnight. The membrane was blocked by 5% non-fat milk in PBS for 1 h at room temperature, followed by incubation with primary antibodies at 4 °C overnight. Primary antibodies used were listed in Table 2. Secondary antibodies were applied with a dilution at 1:5000. The membrane was washed 3 times and rinsed in TBST. Protein blots were detected with ECL substrates (Bio-rad), imaged for colorimetric detection.

Immunohistochemistry and cell counting

Brains were collected and processed to immunostaining as described in chapter 2. Target cells were counted by the same method as described in chapter 2. To quantify mTORC1 activation in total NSCs, every sixth brain sections covering the whole hippocampus were immunostained against NSC marker and pS6. The number of double positive cells were counted in all the sections, and NSCs were

counted in three epicentral sections. The cell density was calculated by dividing cell number by the volume of GCL. To quantify mTORC1 activation in total proliferating NSCs, every sixth brain sections covering the whole hippocampus were immunostained against BrdU, NSC marker, and pS6. The numbers of triple positive cells, and BrdU and NSC marker double positive cells were counted respectively. The cell density was calculated by dividing cell number by the volume of GCL. To evaluate the status of individual RGL NSCs, every sixth brain sections covering the whole hippocampus were immunostained against BrdU, GFP, and pS6. The numbers of GFP^+ $pS6^-BrdU^-$, $GFP^+pS6^+BrdU^-$, $GFP^+pS6^+BrdU^+$, and $GFP^+pS6^-BrdU^+$ were counted respectively, and the percentage of each cell type was calculated by dividing cell number by the number of total GFP^+ cells.

Statistical analysis

Quantification of target cells was shown as average ± standard deviation. Data were analyzed via the appropriate type of analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. Statistical analysis was conducted via SPSS software (IBM Corporation, Armonk, NY). Significance was set at $p<0.05$.

Results

mTORC1 signaling is activated in the hippocampus after TBI

NSCs have been widely reported to respond to various types of brain injuries, including stroke, seizure, and TBI (Dash et al., 2001, Kernie et al., 2001, Braun et al., 2002, Chirumamilla et al., 2002, Rice et al., 2003, Ramaswamy et al., 2005, Gao et al., 2009a, Zheng et al., 2013, Sun et al., 2005, Parent et al., 1997, Yagita et al., 2001, Parent et al., 2002, Yamashita et al., 2006). Following TBI, enhanced NSC proliferation in the hippocampus has been consistently observed regardless of animal model and injury model (Dash et al., 2001, Kernie et al., 2001, Braun et al., 2002, Chirumamilla et al., 2002, Rice et al., 2003, Ramaswamy et al., 2005, Gao et al., 2009a, Zheng et al., 2013, Sun et al., 2005). It was further demonstrated that quiescent NSC is the subgroup that is mainly activated by TBI (Gao et al., 2009a). However, the molecular mechanism of the phenomenon remains elusive, impeding the development of interventions aimed at promoting neurogenesis by further enhancing NSC proliferation post-trauma. Mammalian target of rapamycin (mTOR) signaling pathway, especially mTOR complex 1 (mTORC1), is known to be indispensable in NSC activity regulation in embryonic (Magri et al., 2011), neonatal (Hartman et al., 2013), adult (Paliouras et al., 2012), and aging rodents (Romine et al., 2015, Paliouras et al., 2012), its activation has also been reported post-trauma in the hippocampus (Chen et al., 2007, Park et al., 2012), so I proposed that mTORC1 signaling mediates TBI-enhanced NSC proliferation.

To demonstrate this hypothesis, I induced moderate TBI in adult mice by a CCI injury model and began with evaluation on mTORC1 signal activation in the whole hippocampus at different time points post-injury. Adult mice were sacrificed after sham surgery or at 4 h, 24 h, 48 h, 72 h, and 1 week after CCI. Ipsilateral hippocampi were subjected to immunoblotting. The total amount of ribosomal protein S6 (S6, a widely-used marker for mTORC1 activation (Laplante and Sabatini, 2012), Figure 3.1 A), and its phosphorylated form were examined, respectively. After trauma, the total S6 was elevated rapid at 4 h post-injury, reached the peak at 24 h, and maintained at a high level at 48 h and 72 h, while returned back to normal level at 1 week after injury (Figure 3.1 B). Meanwhile, the amount of activated S6, phosphorylation level (pS6), was dramatically increased at 4 h, further elevated at 24 h, maintained at high level at 48 h, remained slightly higher than sham level at 72 h and returned back comparable to baseline at 1 week (Figure 3.1 C). Together, the data suggested mTORC1 signal was activated in the hippocampus mainly at 4 h, 24 h, and 48 h after TBI.

mTORC1 signaling is activated in the hippocampal dentate gyrus after TBI

To assess the temporal-spatial profile of mTORC1 activation in the hippocampal dentate gyrus (HDG), adult mice were sacrificed after sham surgery or at 4 h, 24 h, 48 h, 72 h, and 1 week after CCI. The epicentral section from each animal was subjected to immunostaining against pS6. A limited number of pS6-positive cells were observed in sham animals, mainly in the granule cell layer (GCL) and few in the hilus, indicating a baseline of mTORC1 activity in the HDG (Figure 3.2 A).

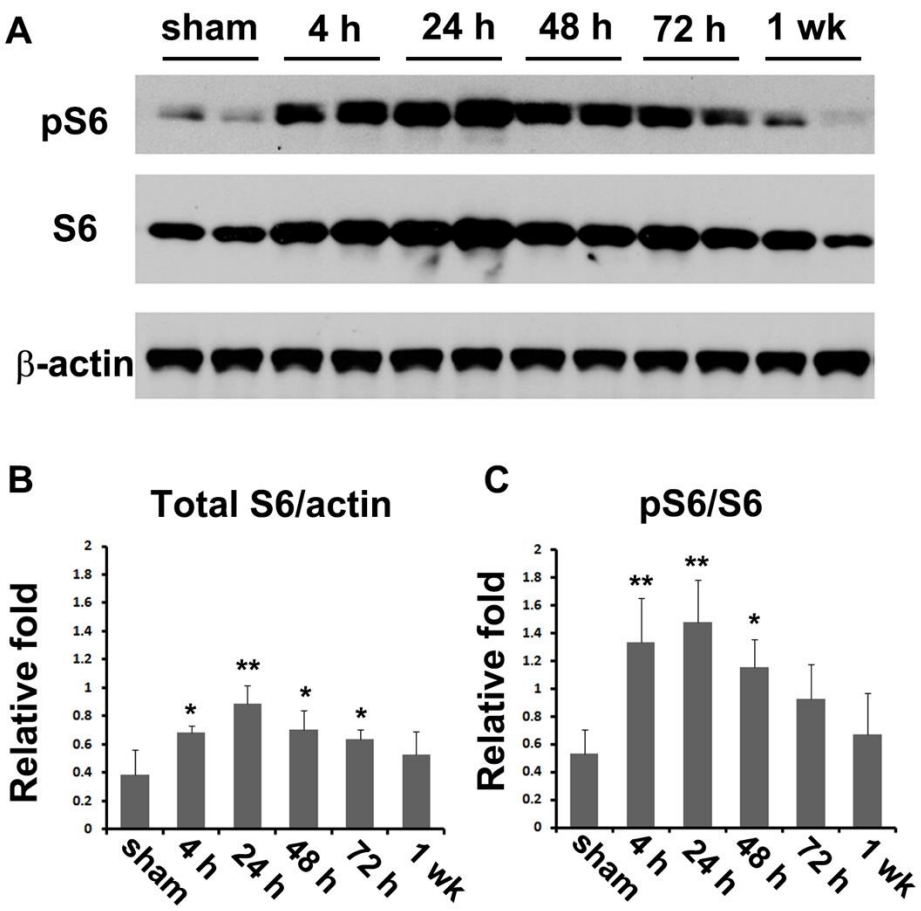


Figure 3.1: TBI activates mTORC1 signaling in the hippocampus.

Mice received a moderate controlled cortical impact (CCI) at the age of 9 weeks and were sacrificed after sham injury or at 4 h, 24 h, 48 h, 72 h, and 1 week after injury (n=3 for each group). (A) Immunoblotting with antibodies against phosphor-ribosomal protein S6 (pS6), ribosomal protein S6 (S6), and β-actin shows mTORC1 signaling activation in the hippocampus. (B, C) Quantification of blots shown in panel A (* $p<0.05$, ** $p<0.01$). Data contributed by Dr. Lin Xu in Chen lab.

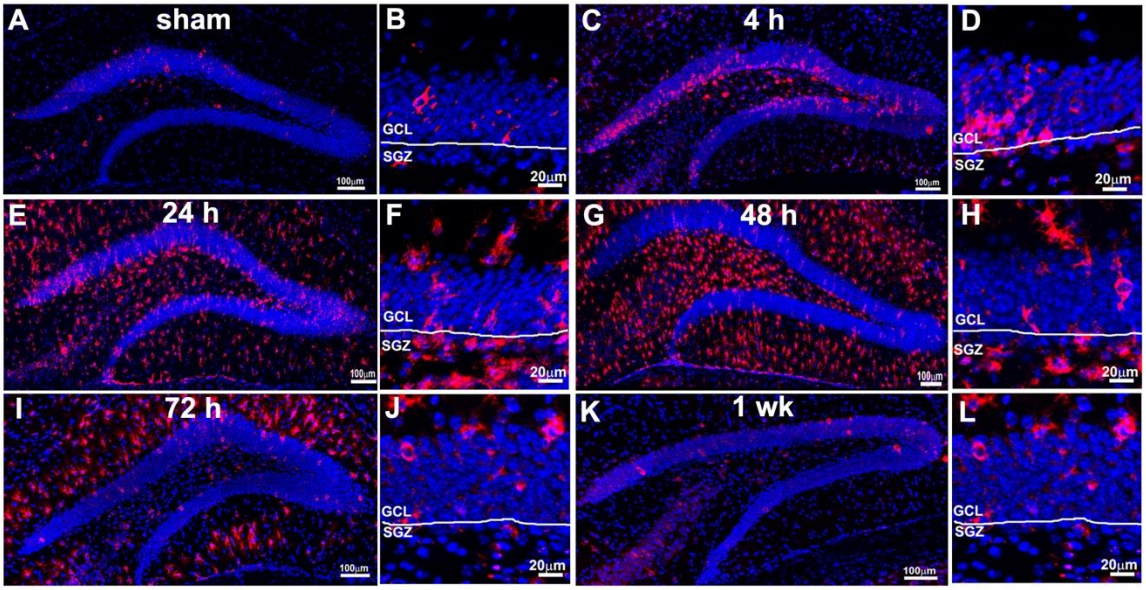


Figure 3.2: TBI activates mTORC1 signaling in different subregions of the hippocampus.

Mice received a moderate controlled cortical impact (CCI) at the age of 9 weeks and were sacrificed after sham injury or at 4 h, 24 h, 48 h, 72 h, and 1 week after injury (n=3 for each group). (A-L) Immunostaining against pS6 (red) shows mTORC1 signaling activation in the hippocampal dentate gyrus after sham surgery (A), and 4 h (C), 24 h (E), 48 h (G), 72 h (I), and 1 week (K) after CCI, and in the subgranular zone at corresponding time points (B, D, F, H, J, L, respectively. DAPI staining shows the structure of hippocampal dentate gyrus.

Following TBI, I observed a wave of mTORC1 activation in the HDG starting no later than 4 h and maintaining a high level of activation at least until 72 h, while returning to sham level at 1 week post-injury (Figure 3.2 C, E, G, I, K).

Meanwhile, mTORC1 activation showed different patterns in terms of signal location in each HDG subregions at different time points. At 4 h post-trauma, increased pS6-positive cells were predominantly restricted in the GCL (Figure 3.2 C), indicating a rapid response in granule neurons to the initial insult. At 24 h post-trauma, the pS6-positive cells further accumulated not only in the GCL but also vastly extended to the molecular layer (ML) and hilus (Figure 3.2 E). At 48 h following CCI, the pS6 signal reached the peak, and positive cells were mainly detected in the ML and hilus but few in the GCL (Figure 3.2 G), implying strong mTORC1 activation in reactive glia, highly likely reactive microglia, at this time point (unpublished data). At 72 h post-trauma, mTORC1 activation started decreasing, and the remaining positive cells were mostly in the ML (Figure 3.2 I), which were largely attributed to reactive astrocytes based on unpublished data. At 1 week after TBI, the pS6 signal was back to sham level and again showed sporadic activation mainly in the GCL (Figure 3.2 K). Collectively, I detected a time-dependent and location-shift mTORC1 activation pattern in the HDG following TBI, indicating mTORC1 involvement in diverse responses to CCI in multiple cell types.

Time-course of mTORC1 signaling activation in the SGZ after TBI

To further examine whether mTORC1 is activated in the NSCs after TBI, I focused on pS6 signal in the SGZ, where adult NSCs reside in the hippocampus (Faigle and Song, 2013). In sham animals, I again observed pS6-positive cells were rare and mainly located in the GCL (Figure 3.2 B). At 4 h post-trauma, increased pS6 signal was seen primarily in the GCL but not SGZ (Figure 3.2 D). At 24 h post injury, a dramatic increase of pS6-positive cells showed up in the SGZ (Figure 3.2 F), as well as in the ML and hilus. At 48 h following CCI, pS6-positive cells in the SGZ decreased compared with 24 h, but was still more than sham level (Figure 3.2 H). At 72 h and 1 week post-trauma, mTORC1 activation in the SGZ was limited and comparable to sham animals (Figure 3.2 J, L). Taken together, my data enabled me to narrow down activation of mTORC1 in the SGZ predominantly to 24 h and 48 h after initial injury, suggesting the potential time course of mTORC1 activation in the NSCs. The period of mTORC1 activation correlates with TBI-enhanced NSC proliferation (Gao et al., 2009a), indicating possible involvement of mTORC1 activity in TBI-enhanced NSC proliferation.

TBI activates mTORC1 signaling in the NSCs

To accurately evaluate whether mTORC1 is activated in the NSCs, I co-labeled pS6 with NSC marker. Additionally, I took advantage of a Nestin-GFP transgenic mouse, in which NSCs ectopically express green fluorescent protein (GFP) (Gao et al., 2009a, Mignone et al., 2004). I hardly observed NSCs co-staining with an antibody to pS6 in the SGZ of sham-treated animals (Figure 3.3 A-C).

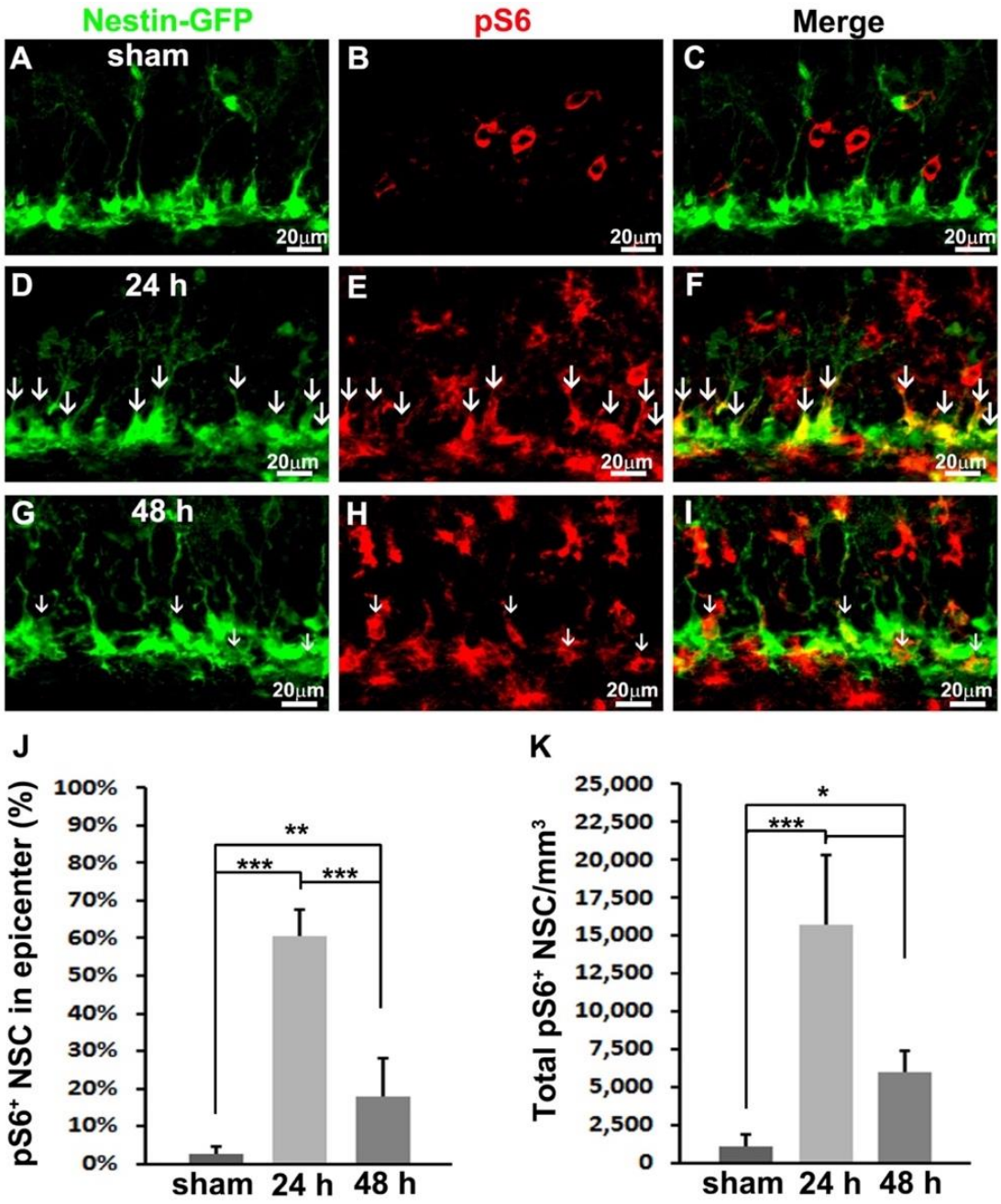


Figure 3.3: TBI activates mTORC1 signaling in NSCs.

Mice received a moderate controlled cortical impact (CCI) at the age of 9 weeks and were sacrificed at 24 h, 48 h after injury as well as after sham injury (n=5 for each group). (A-I) Immunostaining with antibodies against GFP (green) and phosphor-ribosomal protein S6 (pS6, red) shows mTORC1 signaling activation in

neural stem cells (indicated by white arrows) after sham surgery (A-C), 24 h (D-F), and 48 h (G-I) after CCI in the subgranular zone. (J) Quantification of total pS6-positive NSCs after sham surgery, and 24 h and 48 h after CCI, respectively. (K) Quantification of ratio of pS6-positive NSCs in the epicenter after sham surgery, and 24 h and 48 h after CCI, respectively (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Quantification illustrated that 2.6 ± 2.1% of NSCs were pS6 positive at the epicenter (Figure 3.3 J). When I further examined NSCs across the whole hippocampus, there were only 1,096 ± 805 /mm^3 of total NSCs in the whole hippocampus co-labeled with pS6 (Figure 3.3 K). These results confirmed my prior notion that basal mTORC1 activity in the NSCs is very low in the sham animals.

At 24 h after injury, the number of pS6-positive cells in the SGZ were dramatically increased (Figure 3.3 E) and largely labeled NSCs (Figure 3.3 D-F, indicated by white arrows). Quantification showed mTORC1 is active in 60.7 ± 6.8% of total NSCs in the epicenter (Figure 3.3 J). Totally, mTORC1 signaling is active in 15,750 ± 4,620/mm^3 NSCs across the whole hippocampus (Figure 3.3 K), indicating a dramatic 15-fold increase compared to the sham animals.

At 48 h after TBI, the number of pS6-positive cells was still very high (Figure 3.3 H), while labeling in NSCs decreased (Figure 3.3 G-I, indicated by white arrows). At the epicenter, the percentage of mTORC1 positive NSCs also dropped to 17.7 ± 10.4% ($p<0.001$ vs. 24 h, $p=0.017$ vs. sham, Figure 3.3 J). The number of NSCs with active mTORC1 in the whole hippocampus rapidly decreased to 5,987 ± 1,348/mm^3 ($p<0.001$ vs. 24 h), but was still much higher than basal level ($p=0.044$ vs. sham, Figure 3.3 K). Collectively, I observed a rapid and robust activation of mTORC1 in NSCs at 24 h after TBI and lasted at least 48 h following trauma.

TBI activates mTORC1 signaling in the proliferating NSCs

Previously, my colleagues illustrated that TBI transiently promoted NSCs proliferation at around 24-48 h post-injury (Gao and Chen, 2013). The molecular mechanisms of TBI-enhanced NSC proliferation are largely elusive. The period of mTORC1 signaling activation correlates well with NSC proliferation after TBI, a strong indication that mTORC1 signaling activation is involved in TBI-induced NSC proliferation. Thus, I further assessed whether mTORC1 signaling is activated in the proliferating NSCs. A dose of BrdU injection (i.p., 100 mg/kg) was given at 4 h prior to sacrifice (Figure 3.4 A), pulse-labeled the proliferating cells during this 4 h period. A series of every sixth section were processed for triple immunostaining to assess the mTORC1 activity in proliferating NSCs.

In sham-treated animals, only 2,320 ± 513/mm^3 NSCs are proliferating, pulse-labeled by BrdU (Figure 3.4 B, C, E, indicated by white arrowheads, and Figure 3.4 N). Among the proliferating NSCs, only 49 ± 40/mm^3 are pS6 positive (Figure 3.4 O), representing merely 1.9 ± 1.2% of total proliferating NSCs that were pS6 positive (Figure 3.4 Q). These data indicate that only a small proportion of NSCs are proliferating in the hippocampus of mice receiving sham-surgery, and the activity of mTORC1 signaling at these proliferating NSCs is extremely low.

At 24 h after moderate CCI injury, 3,070 ± 658/mm^3 NSCs were proliferating in the hippocampus (Figure 3.4 F, G, I, indicated by both white arrowhead and arrow, and Figure 3.4 N). However, TBI significantly activated mTORC1 signaling in the hippocampus (Figure 3.4 H). Of the proliferated NSCs,

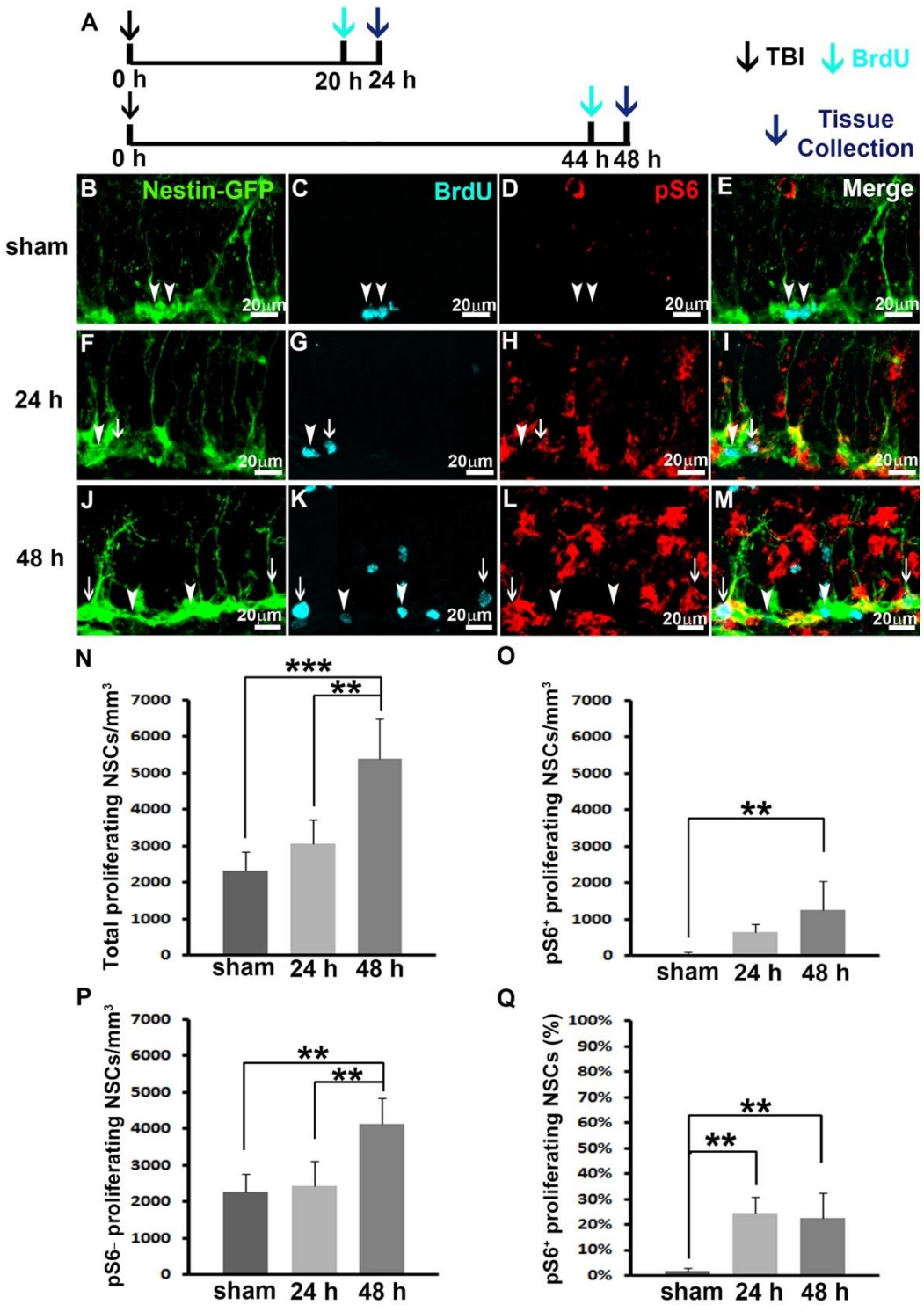


Figure 3.4: TBI activates mTORC1 signaling in proliferating NSCs.

Mice received a moderate controlled cortical impact (CCI) at the age of 9 weeks and were sacrificed at 24 h, 48 h after injury as well as after sham injury (n=5 for

each group). A dose of 5-bromo-2'-deoxyuridine (BrdU) was administered 4 h prior to perfusion. (A) Schematic shows experimental strategy. (B-M) Immunostaining against GFP (green), BrdU (cyan) and phosphor-ribosomal protein S6 (pS6, red) shows proliferating neural stem cells with (indicated by white arrows) or without (indicated by arrowheads) mTORC1 signaling activation in sham animals (B-E), 24 h (F-I), and 48 h (J-M) after CCI in the subgranular zone. (N-P) Quantification of total proliferating NSCs (N) total pS6-positive proliferating NSCs (O) and total pS6-negative proliferating NSCs (P) after sham surgery, and 24 h and 48 h, respectively. (Q) Percentage of pS6-positive proliferating NSCs after sham surgery, and 24 h and 48 h after CCI, respectively (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

649 ± 229/mm^3 or 24.7 ± 6.2% were pS6 positive (Figure 3.4 F-I, pointed out by white arrow, and Figure 3.4 O and Q), while the rest of the proliferating NSCs were pS6-negative (Figure 3.4 F-I, pointed out by white arrowhead). These results suggest that TBI did not dramatically alter NSC proliferation at 24 h compared with sham animals (p=0.328, Figure 3.4 N), consistent with previous report (Gao and Chen, 2013). In contrast, TBI promoted a 13-fold increase of mTORC1 activation in the proliferating NSCs at 24 h after TBI (p=0.001, Figure 3.4 Q).

At 48 h after receiving moderate CCI injury, there are 5,396 ± 1,092/mm^3 NSCs were proliferating in the hippocampus (Figure 3.4 J, K, M, indicated by both arrowheads and arrows, and Figure 3.4 N). TBI dramatically increased NSC proliferation in the hippocampus at this time point (p<0.001), agreeing with previous report that TBI transiently promoted NSC proliferation at 48 hours after TBI (Gao and Chen, 2013). TBI also significantly promoted the mTORC1 signaling in the proliferating NSCs (1,260 ± 798/mm^3, p=0.005 vs. sham, Figure 3.4 J-M, pointed out by white arrows, and Figure 3.4 O). These data indicated that TBI significantly promotes NSC proliferation (Figure 3.4 N) and enhanced mTORC1 activity in the proliferating NSCs at 48 h after injury (Figure 3.4 O, Q). Together, I observed a dramatically increased level of mTORC1 activation in proliferating NSCs at 24 h and 48 h after TBI, while TBI transiently promotes NSC proliferation beginning at 48 hours after TBI. The sequence of mTORC1 activation and increased NSC proliferation strongly indicate the possible

involvement of the mTORC1 signaling activation in TBI-enhanced NSC proliferation.

Inhibition of mTORC1 signaling eliminates TBI-enhanced NSC proliferation

To further confirm mTORC1 activation is required for TBI-enhanced NSC proliferation, I treated TBI injured animals with rapamycin, a well-established mTORC1 inhibitor and then evaluated NSC proliferation at 48 h after TBI. To fully block mTORC1 activity, 4 rapamycin (i.p., 10 mg/kg) or vehicle injections were given at 12 h, 24 h, 36 h, and 44 h after TBI or sham surgery. Right after the last rapamycin injection, a dose of BrdU (i.p.,100 mg/kg) was delivered to label cell proliferation, and then animals were perfused at 48 h after injury (Figure 3.5 A).

To evaluate mTORC1 inhibition, the epicentral section from each animal was subjected to pS6 staining. The low level of mTORC1 activation in sham animals was not apparently affected by rapamycin (Figure 3.5 B, D), while the originally strong mTORC1 activation in the HDG was dramatically abolished in TBI injured animals at 48 h after surgery (Figure 3.5 C, E). The pS6 staining demonstrated successful mTORC1 inhibition in the HDG post-trauma. To assess overall cell proliferation, the epicentral section from each animal was processed to BrdU staining. An obvious decrease of BrdU positive cells in the HDG was observed in both rapamycin-treated sham and TBI animals compared with vehicle-treated groups (Figure 3.5 F-I). These data suggest that inhibition of mTORC1 dramatically reduced cell proliferation in the hippocampus.

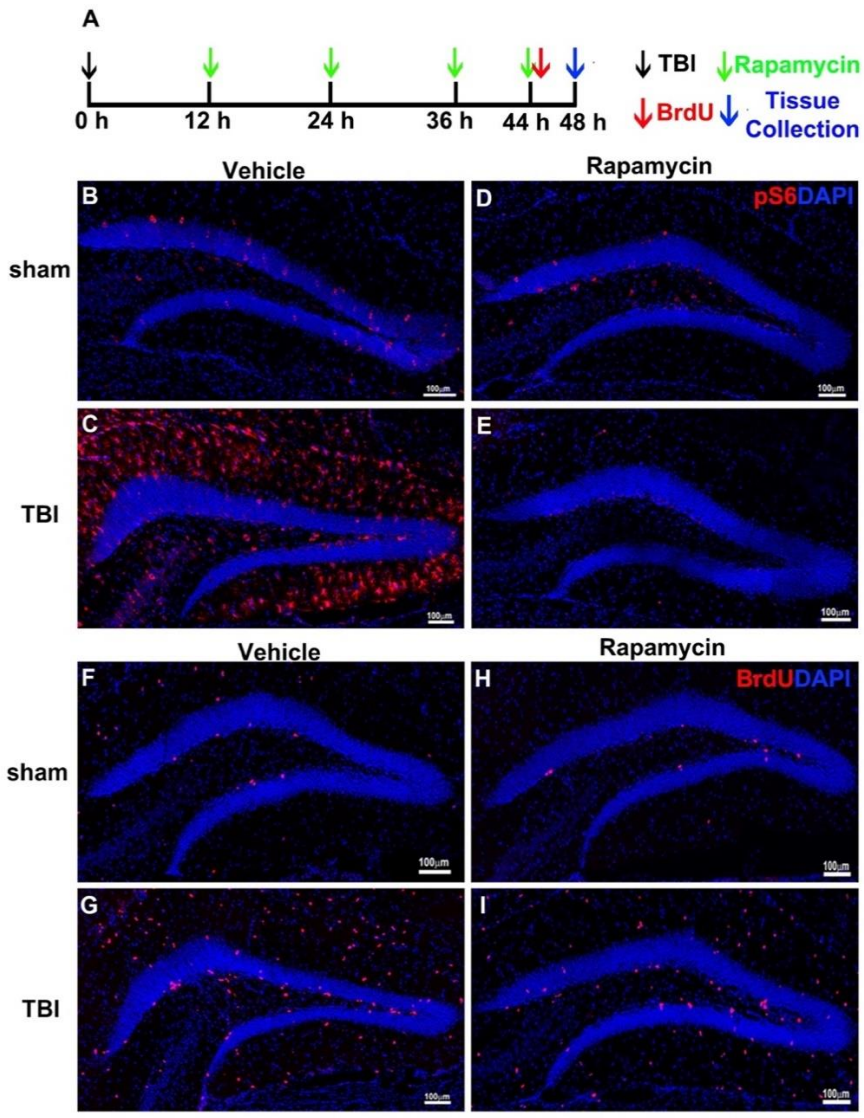


Figure 3.5: Rapamycin treatment inhibits mTORC1 signaling and cell proliferation in the hippocampus following TBI.

(A) Schematic shows experimental strategy. (B-E) Immunostaining with antibody against phosphor-ribosomal protein S6 (pS6, red) shows mTORC1 signaling activity after rapamycin or vehicle in sham animals or 48 h after CCI. (F-I) Immunostaining against BrdU (red) shows cell proliferation in the hippocampal dentate gyrus after rapamycin or vehicle treatment in sham animals or after CCI. DAPI staining shows the structure of hippocampal dentate gyrus.

There are different types of cells, including glia and NSCs, that are proliferating following TBI. To further determine if inhibition on mTORC1 affects NSC proliferation, double immunostaining with NSC markers was performed (Figure 3.6). In sham animals treated with vehicle, there were 1,925 ± 313/mm^3 NSCs proliferating (Figure 3.6 A-C, indicated by arrows, and Figure 3.6 M), which was slightly decreased by rapamycin treatment to 1,436 ± 519/mm^3 without significant difference (p=0.956, Figure 3.6 D-F, and M). After injury, NSC proliferation was dramatically increased to 4,260 ± 1,251/mm^3 (p=0.001 vs. sham + vehicle, Figure 3.6 G-I, and M), while rapamycin treatment abolished the enhanced effect on NSC proliferation (2,375 ± 920/mm^3, p=0.018 vs. TBI + vehicle, Figure 3.6 J-L, and M). Together, my data suggested inhibition on mTORC1 signaling ablates TBI-enhanced NSC proliferation, suggesting that mTORC1 pathway activation is required for TBI-enhanced NSC proliferation.

TBI primarily promotes RGL NSC proliferation but not NPC proliferation

As mentioned earlier, NSCs are the combination of RGLs, the putative multipotent NSCs, and IPCs, the unipotent NPCs (Bond et al., 2015). The two populations are both labeled by GFP expression in the Nestin-GFP transgenic mice, but display distinct morphologies (Mignone et al., 2004). The RGL NSCs hold long radial processes growing through GCL towards ML (Figure 3.7, indicated by white arrow), while NPCs lose their radial processes showing round cell morphologies (Figure 3.7, indicated by white arrowhead) (Mignone et al., 2004). By the morphological differences, I am able to distinguish RGL NSCs from

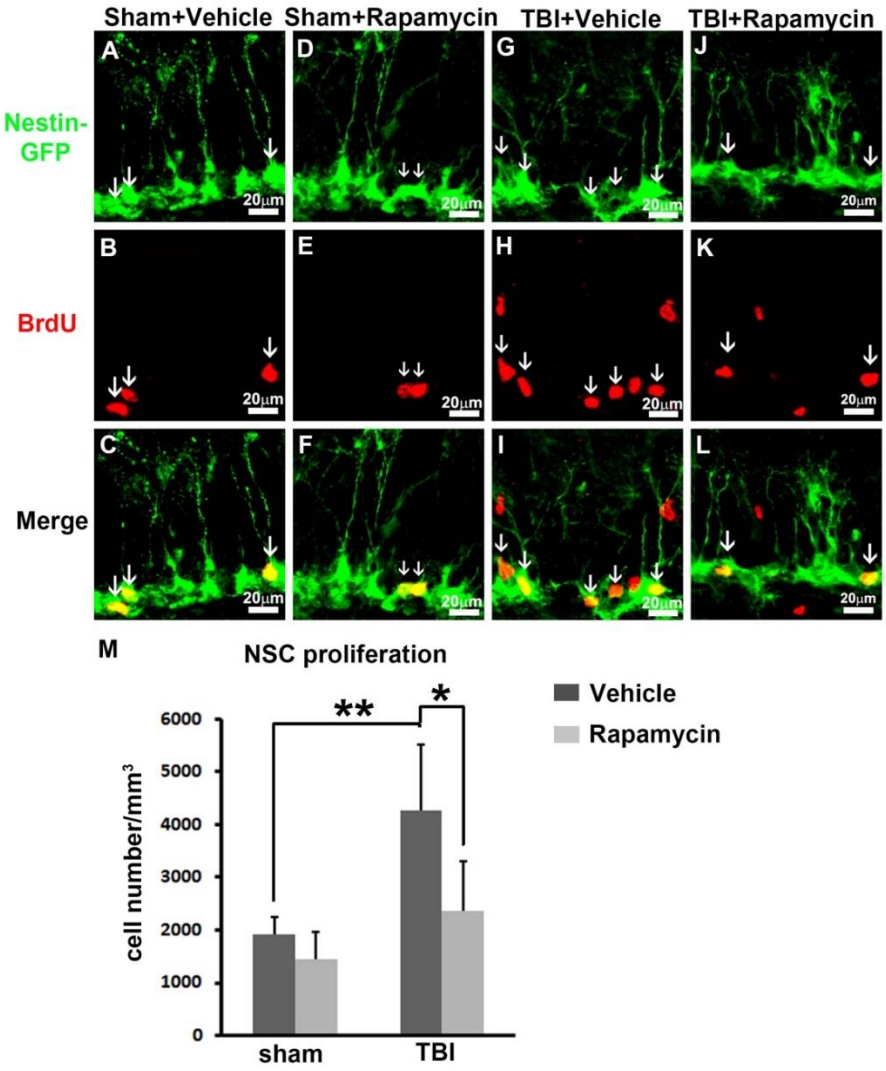


Figure 3.6: Inhibition of mTORC1 signaling ablates TBI-enhanced NSC proliferation.

Mice were treated with the same procedure as shown in Figure 3.5 A (n=5 for each group). (A-L) Immunostaining against GFP (green) and BrdU (red) shows NSC proliferation (indicated by white arrows) in the subgranular zone after rapamycin or vehicle treatment in sham animals and 48 h after CCI. (M) Quantification of NSC proliferation in the subgranular zone after rapamycin or vehicle treatment in sham animals and 48 h after CCI (*p<0.05, **p<0.01, ***p<0.001).

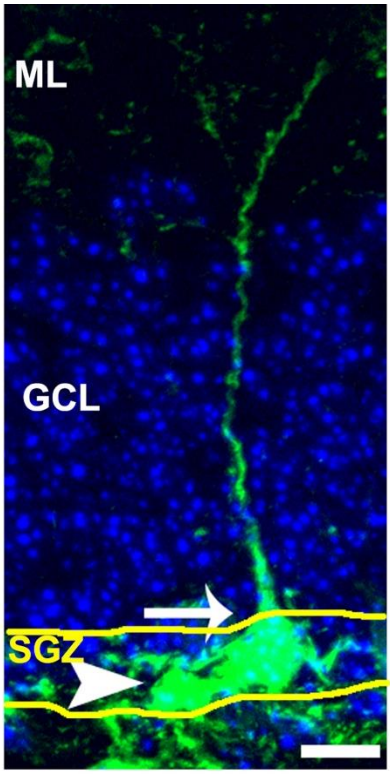


Figure 3.7: Morphological differences of RGL NSCs and NPCs in Nestin-GFP transgenic mouse SGZ.

RGL NSCs show long radial processes growing through GCL towards ML (indicated by white arrow). NPCs show non-radial round morphology without processes (indicated by white arrowhead).

NPCs and study their differences in response to TBI, and the roles mTORC1 might play in it.

I first separated RGL NSC proliferation from NPC proliferation in sham and injured animals at 24 h and 48 h post-trauma (Figure 3.8). In sham animals, the majority of BrdU and GFP double positive cells showed the round cell morphologies without radial processes demonstrating higher baseline of NPC proliferation (Figure 3.8 A-C, indicated by white arrowheads, 1935 ± 178 cells/mm^3, Figure 3.8 J) than RGL NSC proliferation (Figure 3.8 A-C, indicated by white arrow, 456 ± 114 cells/mm^3, Figure 3.8 J), consistent with their regular contributions to adult neurogenesis. At 24 h after TBI, I observed a slight but not significant increase of NPC proliferation (Figure 3.8 D-F, indicated by white arrowheads, 3138 ± 979 cells/mm^3, Figure 3.8 J), and similar phenomenon was also observed for RGL NSC proliferation (Figure 3.8 D-F, indicated by white arrow, 548 ± 122 cells/mm^3, Figure 3.8 J). At 48 h after TBI, I again observed comparable level of NPC proliferation (Figure 3.8 G-I, indicated by white arrowheads, 3161 ± 1032 cells/mm^3, Figure 3.8 J), while the RGL NSC proliferation was dramatically increased to 1346 ± 68 cells/mm^3, 3.0-fold increase compared to sham animals (Figure 3.8 G-I, indicated by white arrows, $p<0.001$ vs. sham, Figure 3.8 J). Together, the data is consistent with a previous study (Gao et al., 2009a), and again proved that it is the RGL NSCs who robustly respond to TBI by increasing their proliferation.

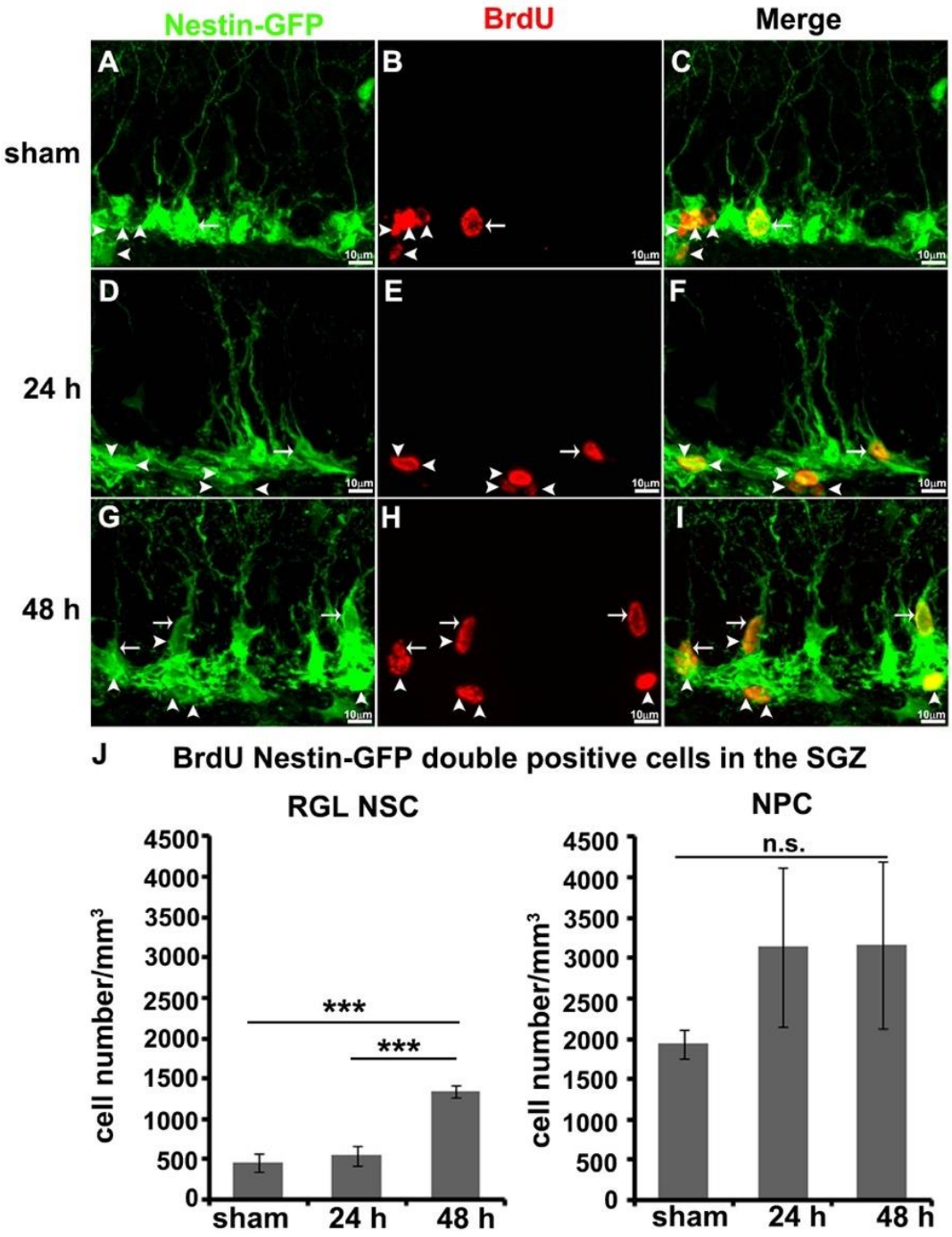


Figure 3.8: TBI primarily promotes RGL NSC proliferation other than NPC proliferation.

Mice received a moderate controlled cortical impact (CCI) and were sacrificed at 24 h, 48 h after injury as well as after sham injury (n=5 for sham, n=3 for 24 h

and 48 h). A dose of 5-bromo-2'-deoxyuridine (BrdU) was administered 4 h prior to perfusion. (A-I) Immunostaining against GFP (green) and BrdU (red) shows proliferating RGL NSCs (indicated by white arrows) and proliferating NPCs (indicated by white arrowheads) in sham animals (A-C), 24 h (D-F), and 48 h (G-I) after CCI in the subgranular zone. (J) Quantification of proliferating RGL NSCs and NPCs after sham surgery, and 24 h and 48 h (***$p<0.001$, n.s. stands for not significant).

mTORC1 is required primarily for RGL NSC proliferation after TBI

Furthermore, I wondered if mTORC1 activation is mediating the different responses of RGL NSCs and NPCs, and is required primarily for RGL NSCs to proliferate after TBI. By the same strategy of separating RGL NSCs from NPCs via morphological differences, I evaluated mTORC1 activity in RGL NSCs and NPCs, respectively (Figure 3.9). In sham animals, I again rarely detected mTORC1 activation in either RGL NSCs or NPCs. There were totally 2.0% ± 1.6% of all RGL NSCs having mTORC1 activation, and the ratio of mTORC1 active NPCs was even smaller (0.9% ± 0.7%, Figure 3.9 A-C, and J). At 24 h after TBI, I again observed dramatic elevation of mTORC1 activity in Nestin-GFP positive cells. The activation of mTORC1 in RGL NSCs was significantly increased to 56.9% ± 5.6% of all RGL NSCs in the hippocampus (Figure 3.9 D-F, pointed by white arrows, $p<0.001$ vs. sham, Figure 3.9 J). For the NPCs, mTORC1 was dramatically activated in 29.0% ± 2.0% of total NPCs in the hippocampus (Figure 3.9 D-F, pointed by white arrowheads, $p<0.001$ vs. sham, Figure 3.9 J), but the level of mTORC1 activation in NPCs is significantly lowered than in RGL NSCs ($p<0.001$, Figure 3.9 J). At 48 h after injury, the mTORC1 activation was again decreased in Nestin-GFP positive cells but still higher compared to sham animals. While the pattern of mTORC1 activation in RGL NSCs versus NPCs was similar with 24 h, in which a larger proportion of RGL NSCs (13.2% ± 4.2%) were still active for mTORC1 activity compared to NPCs (4.7% ± 2.6%, $p=0.002$ vs. RGL NSCs, Figure 3.9 J). Together, my data demonstrated that although mTORC1 was activated in both NSCs and NPCs

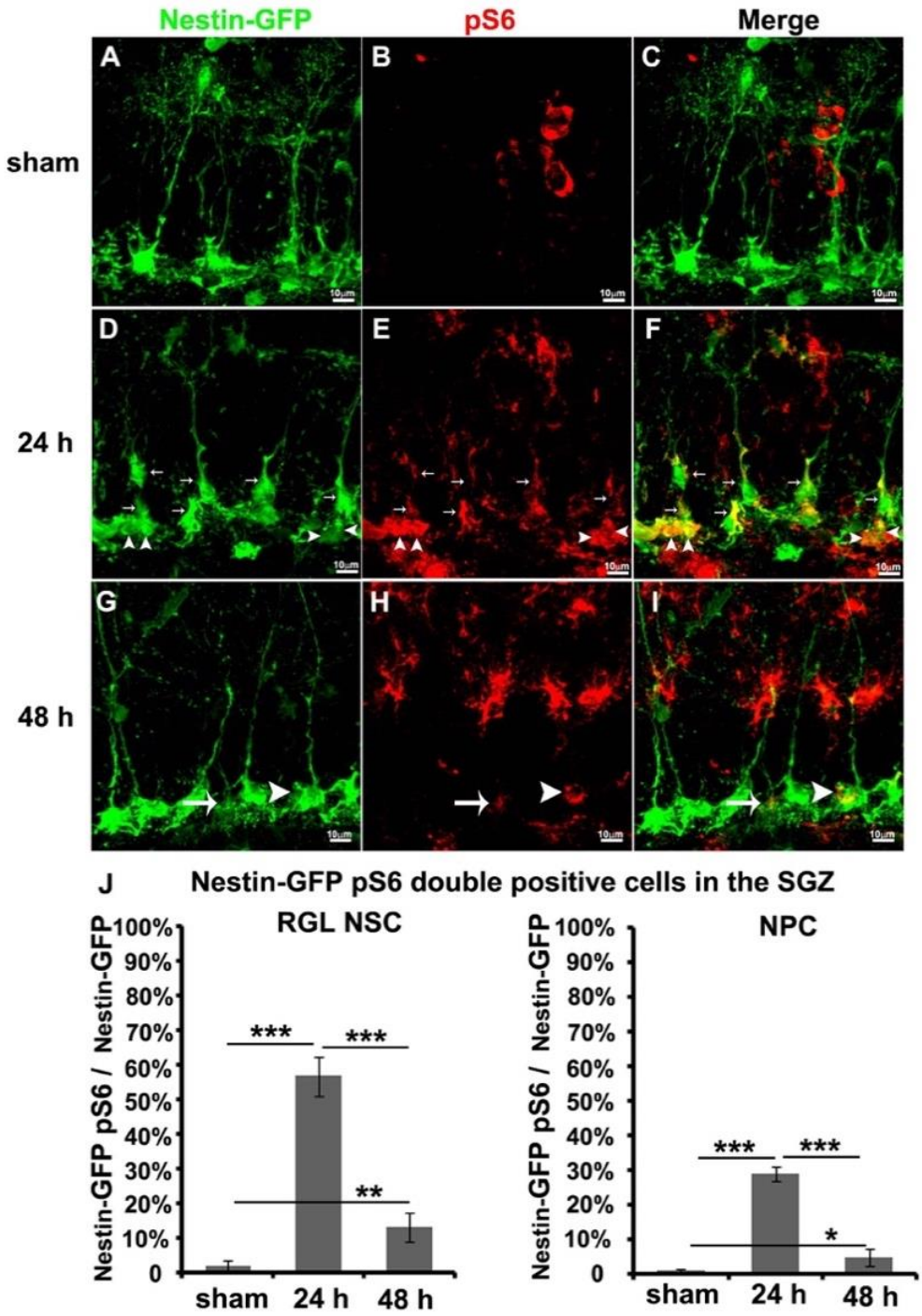


Figure 3.9: TBI primarily activates mTORC1 signaling in RGL NSCs other than in NPCs.

Mice received a moderate controlled cortical impact (CCI) and were sacrificed at 24 h, 48 h after injury as well as after sham injury (n=4 for each group). (A-I)

Immunostaining against GFP (green) and pS6 (red) shows mTORC1 active RGL NSCs (indicated by white arrows) and NPCs (indicated by white arrowheads) in sham animals (A-C), 24 h (D-F), and 48 h (G-I) after CCI in the subgranular zone. (J) Quantification of mTORC1 activation in RGL NSCs and NPCs after sham surgery, and 24 h and 48 h (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

after TBI, the level of mTORC1 activation is significantly greater in RGL NSCs than in NPCs.

To investigate if the differential mTORC1 activation is largely required for TBI-enhanced RGL NSC proliferation but less for NPC proliferation, I assessed how mTORC1 inhibition after TBI affects RGL NSC proliferation and NPC proliferation, respectively. With the same strategy of mTORC1 inhibition by rapamycin administration (Figure 3.5), I detected the baseline of RGL NSC proliferation (Figure 3.10 A-C, indicated by white arrow, 289 ± 73 cells/mm^3, Figure 3.10 J) and NPC proliferation (Figure 3.10 A-C, indicated by white arrowheads, 4680 ± 1444 cells/mm^3, Figure 3.10 J) in sham animals with vehicle treatment. At 48 h after TBI, I again detected a significant increase of RGL NSC proliferation to 1152 ± 118 cells/mm^3 ($p<0.001$ vs. sham + vehicle, Figure 3.10 M), a 3.98-fold increase compared to sham. Whereas, the effects of TBI on RGL NSC proliferation was largely eliminated with mTORC1 inhibition, shown as the RGL NSC proliferation in injured animals with rapamycin treatment is dramatically decreased compared to vehicle treated injury cohorts (448 ± 109 cells/mm^3 vs. 1152 ± 118 cells/mm^3, $p<0.001$, Figure 3.10 J-L, and M), but was still larger than sham controls (448 ± 109 cells/mm^3 vs. 289 ± 73 cells/mm^3, $p=0.026$, Figure 3.10 M). While RGL NSC proliferation in sham animals was not affected by rapamycin (303 ± 76 cells/mm^3, $p=0.831$ vs. sham + vehicle). On the contrary, although the NPC proliferation showed fluctuations, neither TBI insult nor rapamycin treatment significantly altered NPC proliferation ($p=0.054$ for injury, and $p=0.090$ for rapamycin treatment, Figure 3.10 M). Together, the data

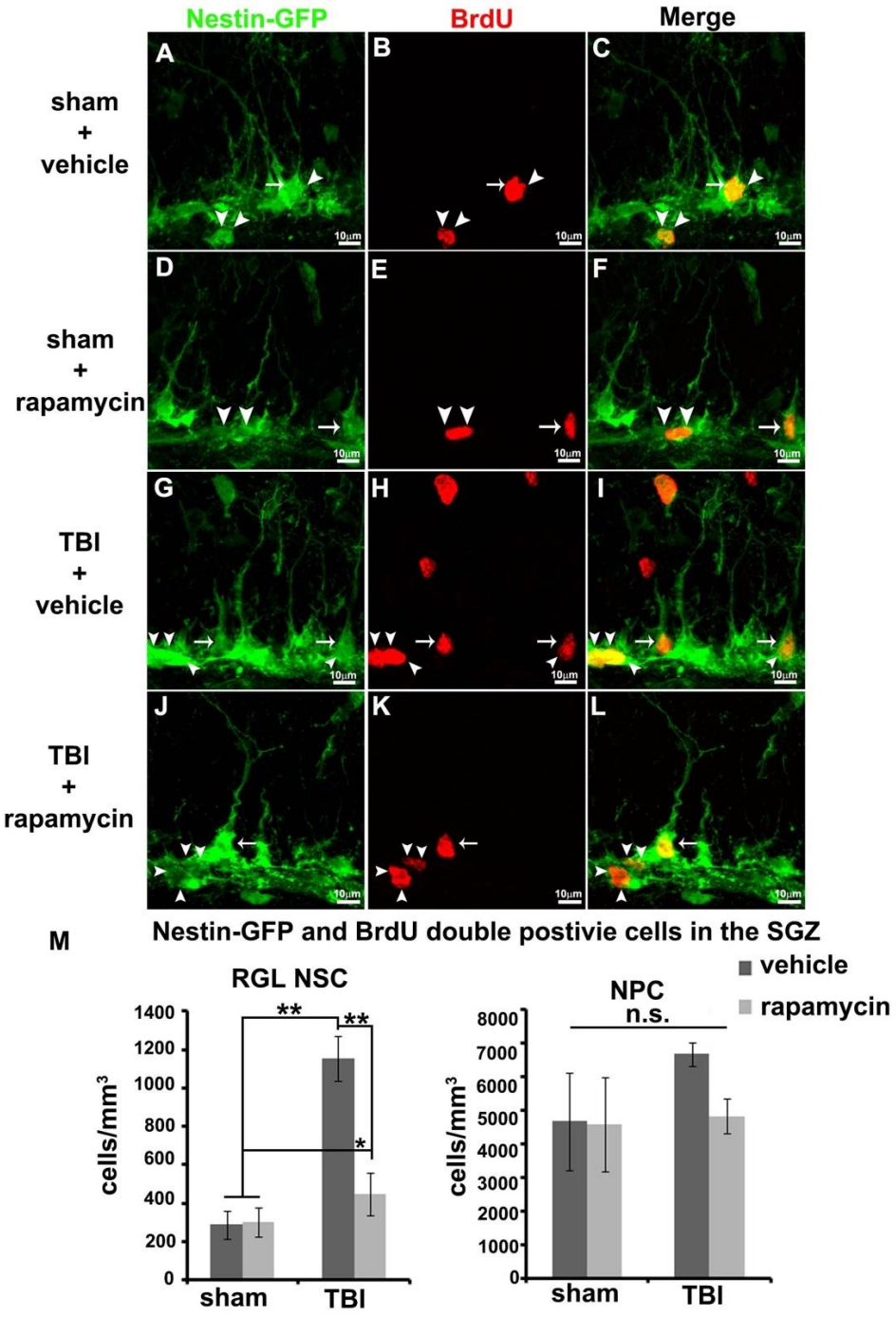


Figure 3.10: Inhibition of mTORC1 signaling primarily ablates TBI-enhanced RGL NSC proliferation other than NPC proliferation.

Mice were treated with the same procedure as shown in Figure 3.5 A (n=5 for sham+vehicle, n=4 for sham+rapamycin, TBI+vehicle, and TBI+rapamycin). (A-L)

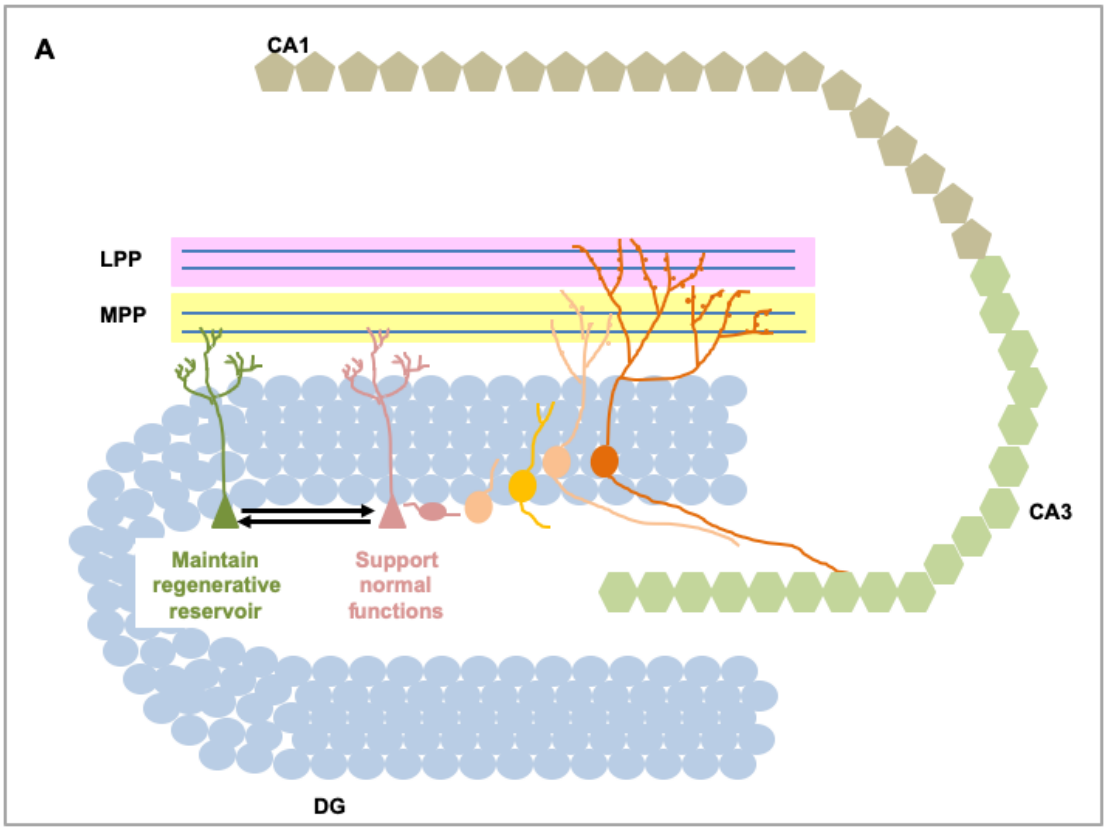
Immunostaining against GFP (green) and BrdU (red) shows RGL NSC proliferation (indicated by white arrows) and NPC proliferation (indicated by white arrowheads) in the SGZ after rapamycin or vehicle treatment in sham animals and 48 h after CCI. (M) Quantification of RGL NSC proliferation and NPC proliferation in the SGZ with rapamycin or vehicle treatment after sham surgery and 48 h after CCI (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

demonstrated that mTORC1 is primarily required for TBI-enhanced RGL NSC proliferation.

mTORC1 primes quiescent RGL NSCs to facilitate proliferation and features a de novo alert state of NSCs

As discussed earlier, RGL NSCs mainly remain quiescent, and only a small proportion is constantly proliferating (Bond et al., 2015). The delicate balance between quiescence and proliferation featured activation is precisely controlled by extrinsic and intrinsic signals, primarily at the step of exit from quiescence (Figure 3.11). My data demonstrated that mTORC1 is primarily mediating TBI-enhanced RGL NSC proliferation, so I wondered how mTORC1 promotes quiescent RGL NSCs to exit quiescence and enter proliferation. To answer the question, I evaluated the status of individual RGL NSCs in sham and injured animals in terms of their mTORC1 activity and proliferative status.

I again combined the Nestin-GFP expression and radial glial morphology to identify each individual RGL NSCs, used pS6 as a marker to assess mTORC1 activity and BrdU label as an indicator of proliferation. In sham animals, I observed that 94.8% ± 0.7% of RGL NSCs were negative for either pS6 or BrdU (Figure 3.12 A-D, indicated by white arrowhead), indicating they are quiescent NSCs (qNSCs), which is consistent with the roles of NSCs to maintain a regenerative reservoir. A small proportion of RGL NSCs were positive for mTORC1 signal but negative for BrdU labeling (Figure 3.12 E-H, indicated by white arrowhead, 2.4% ± 1.1%, Figure 3.12 Q), differentiating them from


A
CA1
LPP
MPP
CA3
Maintain regenerative reservoir
Support normal functions
DG

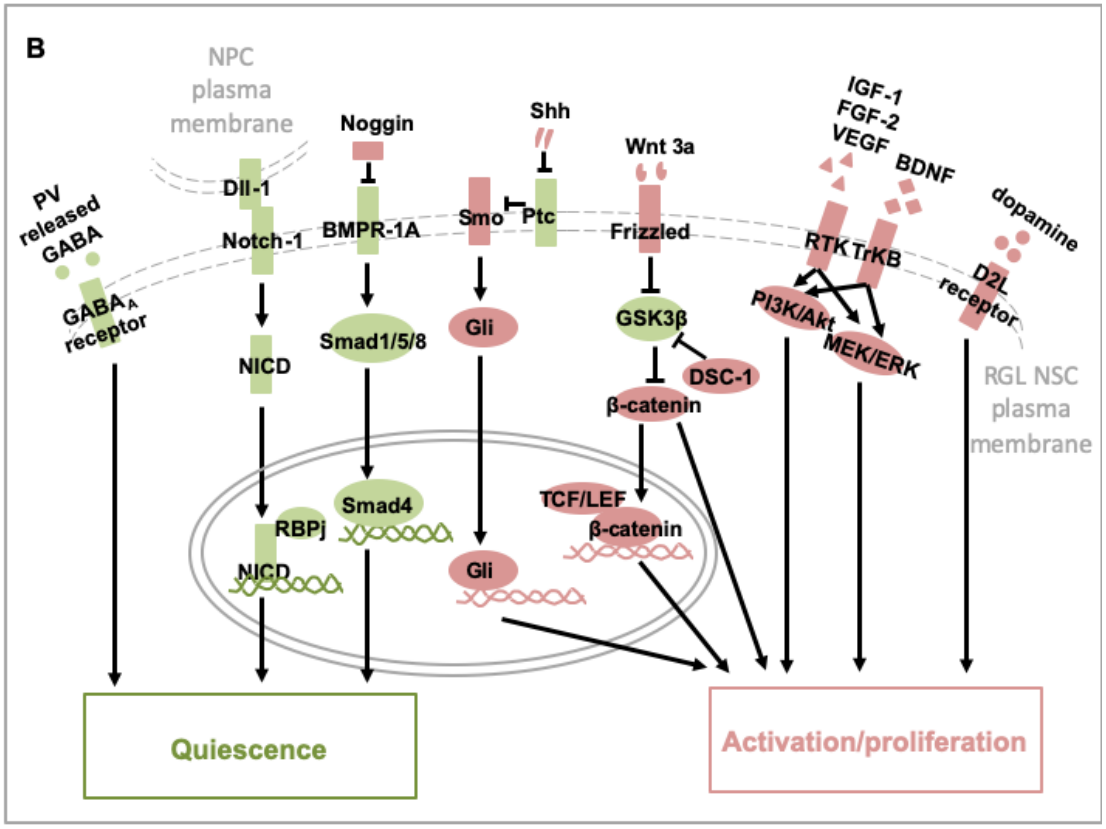

B
NPC plasma membrane
Noggin
Shh
Wnt 3a
IGF-1
FGF-2
VEGF
BDNF
dopamine
PV released GABA
Dll-1
Notch-1
BMPR-1A
Smo
Ptc
Frizzled
RTK
TrKB
D2L receptor
GABA$_A$ receptor
NICD
Smad1/5/8
Gli
GSK3β
DSC-1
β-catenin
PI3K/Akt
MEK/ERK
RGL NSC plasma membrane
Smad4
RBPj
NICD
TCF/LEF
β-catenin
Gli
Quiescence
Activation/proliferation

Figure 3.11: Quiescence and activation balance of RGL NSCs and mechanism of the balance.

(A) RGL NSCs in the SGZ maintains a delicate balance between quiescence and activation. The quiescence maintenance is critical for preserving a regenerative pool of RGL NSCs for lifelong tissue homeostasis and injury repair. The activation is essential for sustaining neurogenesis to support normal cognitive functions, especially learning and memory and emotion regulation. (B) Various extracellular signals together control quiescence and activation balance.

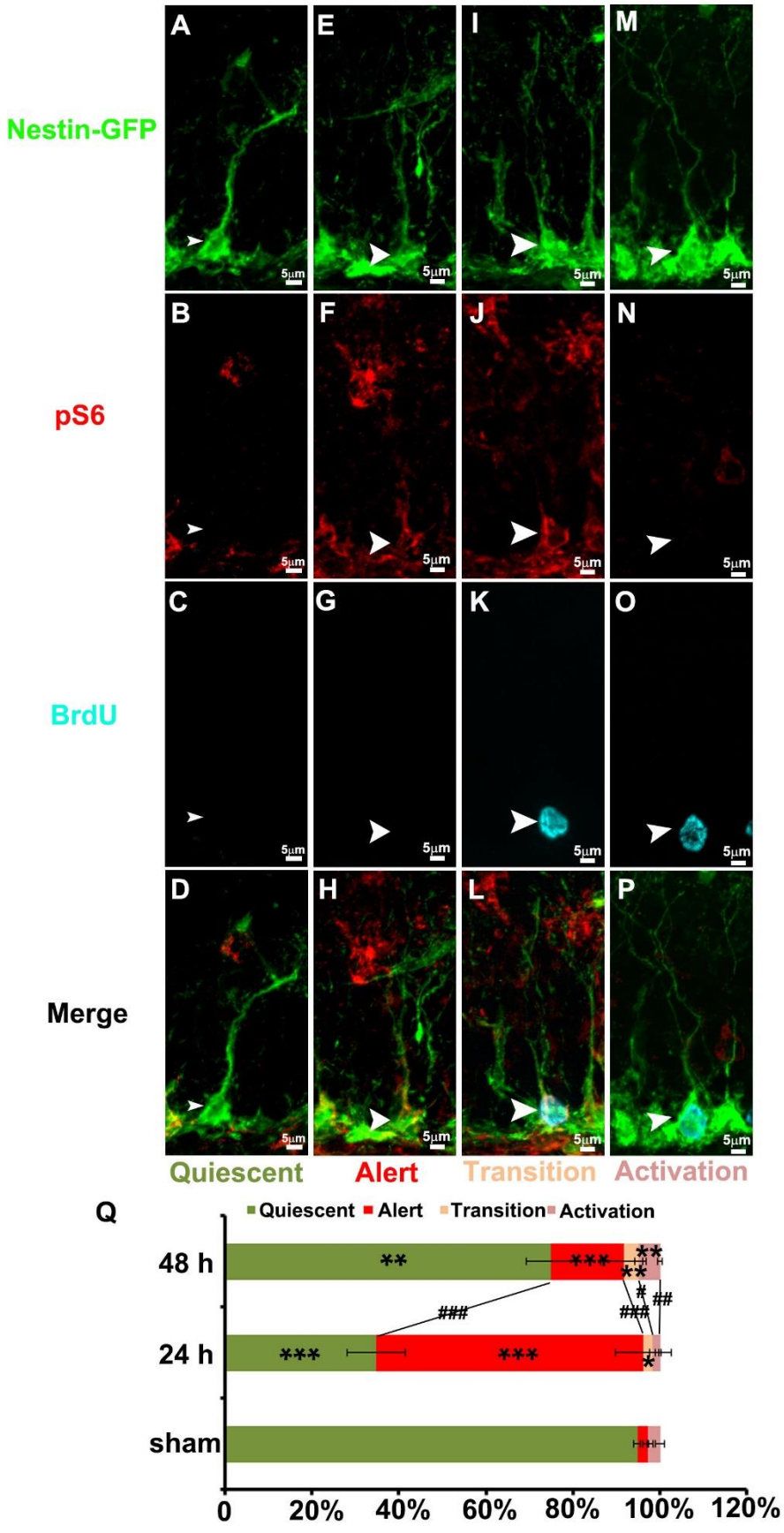

Nestin-GFP
pS6
BrdU
Merge
A
E
I
M
B
F
J
N
C
G
K
O
D
H
L
P
5μm
Quiescent
Alert
Transition
Activation
Q
Quiescent
Alert
Transition
Activation
48 h
24 h
sham
0
20%
40%
60%
80%
100%
120%

Figure 3.12: Activation of mTORC1 signaling features a distinct population of RGL NSCs different from quiescence and BrdU labeled activation.

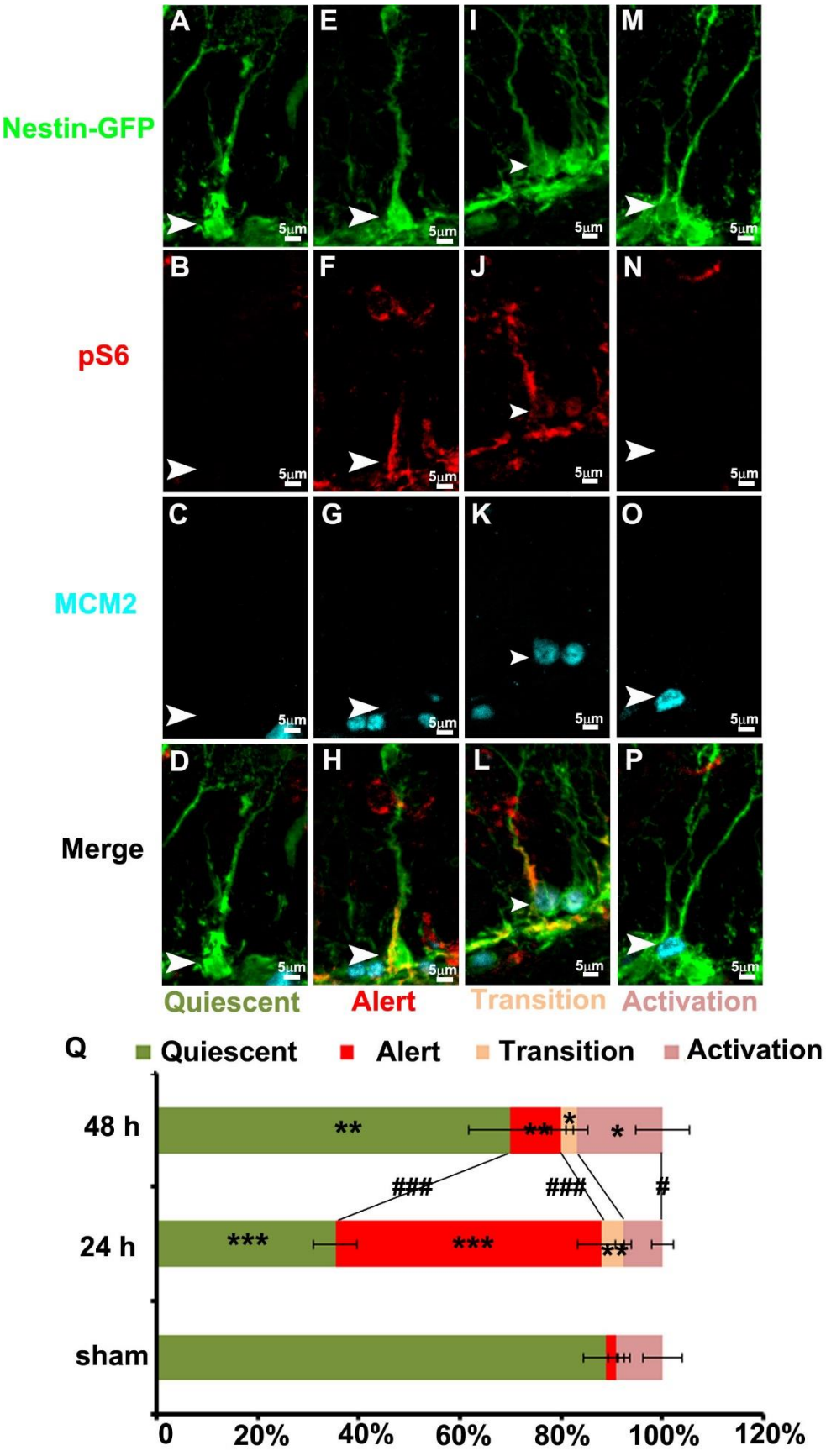
Mice received a moderate controlled cortical impact (CCI) and were sacrificed at 24 h, 48 h after injury as well as after sham injury (n=3 for each group). A dose of 5-bromo-2'-deoxyuridine (BrdU) was administered 4 h prior to perfusion. (A-P) Immunostaining with antibodies against GFP (green), pS6 (red), and BrdU (cyan) shows quiescent RGL NSCs (A-D, indicated by white arrow), alert RGL NSCs (E-H, indicated by white arrow), transition RGL NSCs (I-L, indicated by white arrow), and activation RGL NSCs (M-P, indicated by white arrow). (O) Quantification of RGL NSCs in different statuses after sham surgery, and 24 h and 48 h (*p<0.05, **p<0.01, ***p<0.001, compared to sham. #p<0.05, ##p<0.01, ###p<0.001, compared to 24 h).

quiescent state although they were not proliferating, in which I termed them alert NSCs (alNSCs). Very rare RGL NSCs were seen positive for both mTORC1 activity and BrdU labeling (Figure 3.12 I-L, indicated by white arrowhead, 0.1% ± 0.1%, Figure 3.12 Q), indicating a transient state in which NSCs were activated by starting proliferation and mTORC1 activity was not turned off yet, in which I named them transition NSCs (tNSCs). A small proportion of RGL NSCs were negative for mTORC1 activity and solely positive for BrdU labeling (Figure 3.12 M-P, indicated by white arrowhead, 2.7% ± 1.0%, Figure 3.12 Q), representing a state in which NSCs were actively proliferating when mTORC1 activity is no longer needed for maintaining proliferation. This is the pure proliferation state, which corresponds to active NSCs (aNSCs), who supports learning and memory function through normal neurogenesis (Bond et al., 2015).

At 24 h after injury, I was still able to observe RGL NSCs in these four categories. However, they underwent a dramatic shift between different statuses. The qNSCs ratio dramatically decreased to 34.8% ± 6.7% ($p<0.001$ vs sham, Figure 3.12 Q), in a result, a significant increase was observed for alNSCs (2.4% ± 1.1% in sham to 61.5% ± 6.5% in 24 h, $p<0.001$, Figure 3.12 Q), consistent with previously detected peak of mTORC1 activity in RGL NSCs. Whereas, the percentage of tNSCs was merely increased from 0.1% ± 0.1% to 2.0% ± 0.7% ($p=0.014$, Figure 3.12 Q), and aNSCs were not significantly changed yet ($p=0.138$ vs. sham, Figure 3.12 Q). At 48 h after TBI, I observed that the alNSCs ratio dramatically decreased to 16.7% ± 5.2%, only a quarter of the ratio at 24 h ($p<0.001$ vs. 24 h, Figure 3.12 Q), while the proliferating tNSCs and aNSCs

combination only increased from 2.8% ± 1.1% to 8.4% ± 1.1% (p=0.001 sham vs. 48 h, Figure 3.12 Q). Correspondingly, the qNSC ratio restored to 74.9% ± 5.7% (p<0.001 vs. 24 h, Figure 3.12 Q), indicating the majority of alNSCs returned back to quiescence.

It is well known that BrdU is rapidly metabolized after its introduction into the mouse system and labels proliferating cells in S phase of cell cycle for approximately 4 h (Hayes and Nowakowski, 2000), so I further validated individual RGL NSC status by an endogenous cell cycle maker, MCM2, to more accurately reflect proliferation status. Similarly, I was able to observe RGL NSCs in four different statuses (Figure 3.13 A-P). In sham animals, I detected majority but a slightly less proportion of NSCs were quiescent (88.9% ± 4.6%, Figure 3.13 Q) as detected by BrdU (94.8% ± 0.7%, Figure 3.12 Q), showing negative expressions for either pS6 or MCM2 (Figure 3.13 A-D, indicated by white arrowhead), and relatively same ratio of alNSCs were detected (2.0% ± 1.6%). As a result, a higher ratio of proliferating tNSCs and aNSCs (9.1% ± 3.8%) were observed with the endogenous cell cycle maker MCM2 labeling as I expected. However, the pattern of RGL NSC status distribution was consistent as BrdU labeling, which was shown as majority of qNSC (88.9% ± 4.6%), rare alNSCs (2.0% ± 1.6%), nearly undetectable tNSCs (0.03% ± 0.05%) and small proportion of aNSCs (9.1% ± 3.8%). At 24 h after injury, I again observed a significant decrease of qNSCs ratio (35.4% ± 4.3%, p<0.001 vs. sham, Figure 3.13 Q), along with a dramatic increase of alNSCs proportion (52.5% ± 4.5%, p<0.001 vs. sham, Figure 3.13Q) and slightly increased tNSCs (4.4% ± 1.5%, p=0.002 vs.


Nestin-GFP
pS6
MCM2
Merge
A
E
I
M
B
F
J
N
C
G
K
O
D
H
L
P
5μm
Quiescent
Alert
Transition
Activation
Q
Quiescent
Alert
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Activation
48 h
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0
20%
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60%
80%
100%
120%

Figure 3.13: Activation of mTORC1 signaling features a distinct population of RGL NSCs different from quiescence and MCM2 labeled activation.
Mice received a moderate controlled cortical impact (CCI) and were sacrificed at 24 h, 48 h after injury as well as after sham injury (n=4 for each group). (A-P) Immunostaining with antibodies against GFP (green), pS6 (red), and MCM2 (cyan) shows quiescent RGL NSCs (A-D, indicated by white arrow), alert RGL NSCs (E-H, indicated by white arrow), transition RGL NSCs (I-L, indicated by white arrow), and activation RGL NSCs (M-P, indicated by white arrow). (O) Quantification of RGL NSCs in different statuses after sham surgery, and 24 h and 48 h (*p<0.05, **p<0.01, ***p<0.001, compared to sham. #p<0.05, ##p<0.01, ###p<0.001, compared to 24 h).

sham) and unchanged aNSCs (7.7% ± 2.1%, p=0.628 vs. sham). At 48 h after TBI, a similar result was observed as a dramatic decrease of alNSCs (9.9% ± 2.5%, p<0.001 vs. 24h), with a significant but small increase of proliferating tNSC and aNSC together (20.2% ± 7.1%, p=0.010 vs. sham) and great restoration of qNSC proportion (69.9% ± 8.2%, p<0.001 vs. 24 h).

Collectively, my data with exogenous and endogenous cell cycle markers illustrated the existence of a noteworthy population of NSCs that are mTORC1 active but not proliferating, which was termed alert NSCs (alNSCs) and was dramatically increased by injury transiently at 24 h after injury. The alNSCs showed the capacities to either enter proliferation or return back to quiescence, presumably depending on extra signals. I hypothesized that alNSCs is a *de novo* state of NSCs, different from the classically known quiescence and activation/proliferation, featured by mTORC1 activation. In the alert state, NSCs activate mTORC1 pathway to reversibly exit quiescence and prepare for potential proliferation for tissue homeostasis or injury repair, possibly by elevating overall protein synthesis, cellular metabolism, and/or macromolecule biogenesis (Laplante and Sabatini, 2012). Whereas, they are not guaranteed to necessarily enter proliferation and maintain the option to return to quiescence. Further characterizations on the protein synthesis level, cellular metabolism status, transcriptional activities, and/or gene expression patterns would help distinguish alNSCs from qNSCs and aNSCs and validate its existence as a *de novo* state between quiescence and activation/proliferation.

Discussion

TBI causes tremendous hippocampal cell death, resulting in vast disconnections of neurocircuitries and subsequent neurobehavioral dysfunctions. To date, no FDA-approved drug against neuronal loss caused by TBI is available, and effective neuroprotective or alternatively neural repair approaches are urgently needed. Neural stem/progenitor cells (NSC) in the hippocampus resemble the potential of neuroregeneration in the adult brain and hold great promise for neuronal replenishment post-trauma (Kuhn et al., 1996, Shapiro and Ribak, 2005, Ming and Song, 2005, Zhao et al., 2006). NSC proliferation has been widely reported to increase following TBI (Dash et al., 2001, Kernie et al., 2001, Braun et al., 2002, Chirumamilla et al., 2002, Rice et al., 2003, Ramaswamy et al., 2005, Gao et al., 2009a, Zheng et al., 2013, Sun et al., 2005), the same as generation of mature neurons in some circumstances (Sun et al., 2005, Sun et al., 2007, Wang et al., 2016b). This phenomenon shows the regenerative potential of the adult brain by the neurogenic response of NSCs after TBI. However, the increased NSC proliferation not always results in enhanced neurogenesis (Gao and Chen, 2013, Wang et al., 2016b). Therefore, the innate response is not always adequate to fully compensate for neuronal loss in some cases, which requires approaches to further promote NSC proliferation post-trauma. Unfortunately, the molecular mechanisms of TBI-enhanced NSC proliferation are currently unknown, largely impeding its application.

As a fundamental pathway in all cell types, mTOR signaling is involved in various important cellular events, including cell cycle progression, cell survival,

cell metabolism, and cell growth (Laplante and Sabatini, 2012). Especially in the nervous system, particularly in NSCs, mTOR signaling, specifically mTORC1, is significant for balancing differentiation and self-renewal. It has been reported that in the embryonic stage, constitutive mTORC1 signaling hyperactivity is responsible for deregulated NSCs activity and leads to the development of tuberous sclerosis (Magri et al., 2011). In the postnatal development, mTORC1 activity is also required for NSCs, particularly in the maintenance of the transit amplifying neural progenitor pool (Paliouras et al., 2012). The decline of NSC proliferation in aging rodents can also be restored by activating mTORC1 activity (Romine et al., 2015). Collectively, mTORC1 plays significant roles in regulating NSCs activity, particularly in regulating NSC proliferation. In the current study, I demonstrated that mTORC1 activation is primarily mediating TBI-enhanced RGL NSC proliferation by driving qNSCs out of quiescence and priming them to a *de novo* alert state, and inhibition of mTORC1 activation primarily eliminated injury promoted RGL NSC proliferation. Thus, I identified a molecular target to potentially promote post-traumatic NSC proliferation and subsequent endogenous neurogenesis.

Before I proved its role in mediating post-traumatic NSC proliferation in the current study, activation of mTORC1 signaling in the ipsilateral cortex and hippocampus has been noticed for a while (Chen et al., 2007, Park et al., 2012). In initial studies, mTORC1 was shown to mediate apoptotic neuronal death within hours after TBI, and early inhibition of mTORC1 signaling by rapamycin pre-treatment has been shown to alleviate motor deficits and cognitive impairments

at 3 days post-trauma (Park et al., 2012, Ding et al., 2015). Later on, mTORC1 activation in astrocytes and microglia post-injury was also documented, which supported the idea that the role of mTORC1 in TBI pathogenesis included inducing neuroinflammation and promoting astrogliosis, which could be reversed by rapamycin administration (Ding et al., 2014, Nikolaeva et al., 2015). In addition, the genetic activation of mTORC1 by inactivating upstream negative regulator worsens cognitive performances after TBI in rodents (Rozas et al., 2015). Butler and colleagues additionally pointed out a regulatory role of mTORC1 in post-traumatic neurogenesis and synaptic reorganization, which was potentially involved in post-traumatic epileptogenesis (Butler et al., 2015). The studies supported the idea that mTORC1 activation was considered a deleterious event after TBI.

However, another study instead demonstrated that rapamycin treatment worsened cognitive deficits (Zhu et al., 2014). My colleague's unpublished data also indicates that mTORC1 activation is required for survival of spared neurons in the injured cortex. The exact roles of mTOR in TBI pathophysiology has yet to be determined. In the present study, I focused on the regulatory role of mTORC1 in NSC proliferation after moderate TBI using a CCI model in mice. I illustrated that mTORC1 activation in NSCs in the hippocampus was delayed to after 24 h to 48 h of initial injury compared with 4 h post-trauma in mature neurons as previously reported (Chen et al., 2007, Ding et al., 2015). Inhibition of mTOR by rapamycin abolished TBI-enhanced NSC proliferation in the hippocampus at 48 h in a model of moderate CCI. My study demonstrated a beneficial role of

mTORC1 in neuroplasticity by mediating NSC proliferation after TBI. Although some studies described above indicate that inhibition of mTORC1 activity by rapamycin treatment pre- or post-injury improved motor functions and cognitive outcomes at the acute phase after TBI, my results suggest that the use of rapamycin may come at the expense of compromising neuroregeneration in a long-term manner. On the contrary, enhancing mTORC1 activation may increase neurogenesis and have a beneficial effect in improving learning and memory functions in the long term.

Besides its therapeutic potential as a neural repair treatment after TBI, the role of mTORC1 in NSC activity regulation also sheds light on the principle question of how NSCs maintain balance between quiescence and activation/proliferation. There are at least 2 subtypes of NSCs that can be categorized in the adult hippocampal dentate gyrus. They can be distinguished from each other based on their radial vs. non-radial morphologies, protein marker, such as GFAP expression, and asymmetric vs. symmetric division modes (Seri and Garcia-Verdugo, 2001, Filippov et al., 2003, Bull and Bartlett, 2005, Seaberg and van der Kooy, 2002, Mignone et al., 2004, Encinas et al., 2006, Encinas et al., 2008, Encinas and Enikolopov, 2008). Radial glia-like (RGL) cells are the putative neural stem cells (NSCs). Only a small fraction (1.73 % to 2.81 %) of them can be labeled by BrdU after a 4 hours pulse ((Gao et al., 2009a) and Figure 3.12), implying that they mainly remain quiescent (Mignone et al., 2004, Encinas et al., 2006). Thus, RGL NSCs are consisted of a majority of quiescent neural stem cells (qNSC) and sparse active neural stem cells (aNSCs)

that are proliferating. Functionally, qNSCs maintain stem cell pool, while aNSCs asymmetrically divide to generate small round or oval shape neural progenitor cells (NPCs) (Seri et al., 2001, Bonaguidi et al., 2011). These progeny cells undergo limited rounds of symmetric divisions to generate neuroblasts. They can be labeled by BrdU in high frequency, approximately 14.99% (Gao et al., 2009a). Hence, they are termed as amplifying neural progenitor cells (NPCs) (Mignone et al., 2004, Encinas et al., 2006). My colleagues have previously proved that TBI mainly activates qNSCs (Gao et al., 2009a), and the observation has been again confirmed in the current study (Figure 3.8). The RGL NSCs dramatically increased their proliferation by 3.0-fold at 48 h post-trauma, whereas NPCs slightly but not significantly increase their proliferation at either 24 h or 48 h, indicating the therapeutic potential of specifically RGL NSCs in neuronal replacement represented neural repair. The difference in proliferative responses was further proved to be attributed to mTORC1 signal activation, as the inhibition of mTORC1 signal after TBI primarily eliminates RGL NSC proliferation while left NPC proliferation only marginally affected (Figure 3.10). This leads to the hypothesis that mTORC1 regulates quiescence and activation/proliferation balance of RGL NSCs.

The RGL NSCs differ from NPCs in proliferative profiles partially shown as their unique ability to maintain quiescence. Cellular quiescence is a non-proliferating state in which RGL NSCs are actively and reversibly restrained in the G_0 phase of the cell cycle (Coller, 2011). The maintenance of quiescence, exit from quiescence and initiation of proliferation, and return back to quiescence

of RGL NSCs are tightly regulated to preserve normal tissue homeostasis while also support normal functions (Figure 3.11) (Bond et al., 2015). After TBI, I observed a noticeable population of RGL NSCs featured by mTORC1 signal activation that peaked at 24 h, representing 52.5% ± 4.5% of total NSCs, and I termed them alert NSCs (alNSCs). Whereas, the activation of mTORC1 signal in proliferating NSCs constantly maintained at a low level with no larger than 4.4% ± 1.5% of total RGL NSCs, and I termed them transition NSCs (tNSCs) (Figure 3.13). Despite the large proportion of alNSCs emerged after TBI, the percentage of proliferating tNSCs and aNSCs was only increased from 9.1% ± 3.8% to 20.2% ± 7.1% at 48 h after injury, and qNSCs population exhibited a restoration from 35.4% ± 4.3% to 69.9% ± 8.2% (Figure 3.13). The disparity between alNSCs and proliferating tNSCs / aNSCs populations suggests mTORC1 activation does not directly initiate the proliferation program. The separate time courses and limited overlap between mTORC1 signal and proliferation marker indicate involvement of mTORC1 in cellular events before cell cycle entry. The restoration of qNSCs proportion indicates mTORC1 activation features a reversible state different from quiescence. Collectively, I proposed a model of mTORC1 featured *de novo* state of NSCs, in which mTORC1 activation reversibly drives NSCs out of quiescence into an alert state (Figure 3.14). The alert NSCs can either enter cell cycle or return back to quiescence, possibly both depending on extra regulatory mechanisms. In the alert state, NSCs are different from quiescent and primed by mTORC1 activation, possibly by elevating overall cellular activities, to be prepared for potential proliferation.

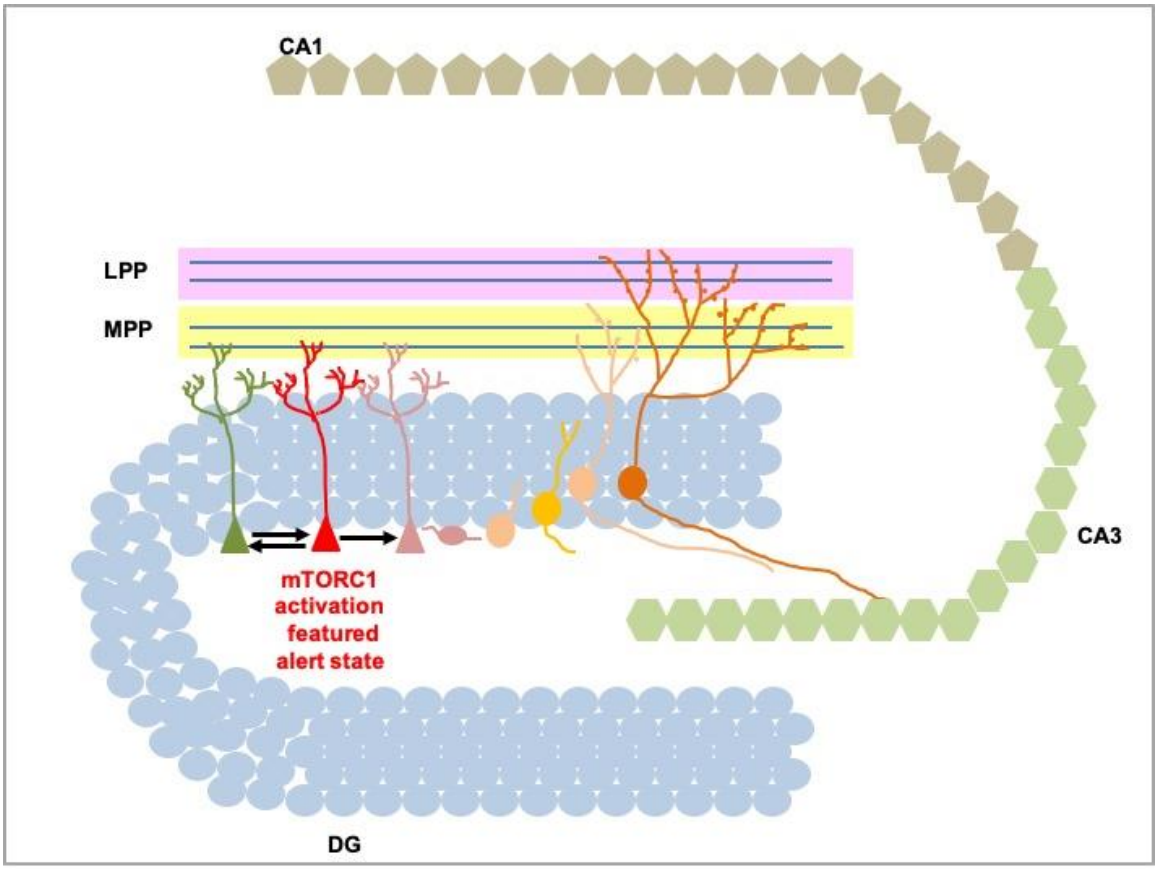


Figure 3.14: Alert RGL NSCs featured by mTORC1 signaling activation. Activation of mTORC1 features a *de novo* state of NSCs, in which I termed alert NSCs. The alert NSCs can either return back to quiescence or initiate proliferation, possibly depending on extra signals.

The alert state between quiescence and activation/proliferation provides a fine-tuning regulatory node for NSCs to modulate their activities to fit the dynamic changes in the environment. The existence of an alert state is supported by single cell transcriptomics studies. Single NSCs from the naïve adult hippocampus and injured SVZ were analyzed and a group of cells showed distinct gene expression profile different from qNSCs and aNSCs (Shin et al., 2015, Llorens-Bobadilla et al., 2015). My study suggests that the distinct NSC state detected in single cell transcriptomic studies corresponds to the alert state I proposed. And the alert state is featured and can be identified by mTORC1 activation. Further characterizations on alNSCs in terms of their protein synthesis rate, cellular metabolism activities, macromolecule biogenesis level, and transcriptional profiles would help define them as a distinct state by characterizing molecular differences such as single cell transcriptomics in qNSCs and aNSCs.

The role of mTORC1 in regulating the transition of stem cell quiescence to activation/proliferation is not limited to the nervous system. It has also been reported in hematopoietic stem cells and muscle stem cells (Yilmaz et al., 2006, Rodgers et al., 2014). The regulation of protein synthesis rate is one of the shared mechanisms of how mTORC1 controls the transition of quiescence to activation/proliferation in different types of stem cells (Signer et al., 2014, Rodgers et al., 2014). Together, mTORC1 might serve as a fundamental regulator of stem cell quiescence and activation/proliferation transition across

different systems. Further characterizations on alNSCs would also shed light on potential mechanisms regulating stem cell activities in other organs.

In conclusion, the body of my works in chapter 3 demonstrated that TBI enhances primarily RGL NSC proliferation by activating mTORC1 signal mainly in RGL NSCs. And mTORC1 promotes RGL NSC proliferation by reversibly driving RGL NSCs out from quiescence to an alert state. This conclusion provides a molecular target that can be potentially utilized to increase post-traumatic NSC proliferation and possibly concomitant post-traumatic neurogenesis. Thus, in chapter 4, I evaluated the effects of a pharmacological approach targeting mTORC1 signaling on cognitive function recovery after TBI and assessed if it functions through activating mTORC1 in NSCs and subsequently increasing post-traumatic neurogenesis.

CHAPTER 4

ASSESSMENT ON EFFECTS OF A PHARMACOLOGICAL APPROACH TARGETING MTORC1 SIGNALING PATHWAY ON COGNITIVE FUNCTION IMPROVEMENT

Hypotheses

1) Ketamine treatment improves spatial learning and memory function after TBI, 2) ketamine treatment enhances mTORC1 signaling in NSCs after TBI, and 3) ketamine treatment promotes post-traumatic neurogenesis.

Introduction

The results in chapter 3 demonstrated that mTORC1 signaling is mediating TBI-enhanced NSC proliferation. Thus, I further tested if a pharmacological approach that targets mTORC1 signaling can promote NSC proliferation after TBI. This would thus enhance post-traumatic neurogenesis and improve cognitive function recovery. Although mTOR, especially mTORC1, has been recognized as a fundamental pathway regulating cell growth, proliferation, migration and survival, development of pharmacological approach mainly focused on its inhibition, mainly because of its active involvement in cancer progression (Laplante and Sabatini, 2012). Activation of mTORC1 is usually achieved by genetic modifications on its upstream negative regulator PTEN and/or TSC1/2 in animal studies (Magri et al., 2011, Park et al., 2008, Liu et al., 2010), which is not translatable to human studies. Although some small molecule inhibitors of PTEN

and combination of growth factors are able to activate mTORC1 activity (Walker et al., 2012, Walker et al., 2019, Liu et al., 2017), they are either not FDA-approved or not able to cross the blood-brain barrier with peripheral delivery. To probe an FDA-approved blood-brain barrier permeable pharmacological approach activating mTORC1, I have chosen to test a sub-anesthetic dose of ketamine treatment in TBI model.

Ketamine was initially synthesized as an NMDA receptor channel blocker in 1962. It has been widely used in clinics for anesthesia and pain management since its FDA approval in 1970. In the past two decades, ketamine has emerged as a therapeutic with rapid anti-depressant effects when used at a sub-anesthetic dose (0.5mg/kg in anti-depressant versus 1-4.5mg/kg in anesthesia) (Berman et al., 2000, Zarate et al., 2006). Mechanistic investigations demonstrated that ketamine dose-dependently activates mTORC1 signaling in the prefrontal cortex in rats, which is required for the rapid anti-depressant effect (Li et al., 2010). The activation of mTORC1 by ketamine only happens at the sub-anesthetic and not the anesthetic dose. This was the first proof of a sub-anesthetic dose of ketamine acting as an mTORC1 activator. My colleagues have used ketamine to activate mTORC1 signaling in aging NSCs. I demonstrated that ketamine promoted the proliferation of NSCs and enhanced neurogenesis in the aging mouse hippocampus (Romine et al., 2015). Thus, I hypothesized that ketamine can activate mTORC1 signaling in NSCs after TBI in young adult mice, which would subsequently enhance post-traumatic neurogenesis, and improve cognitive function recovery. To test the hypothesis, I treated animals with a sub-anesthetic

dose of ketamine at 10mg/kg, and tested spatial learning and memory function of injured and sham animals with or without ketamine treatment. My data demonstrated that ketamine treatment benefited spatial learning but not memory function recovery after TBI. Further histological assessments demonstrated that ketamine treatment can promote mTORC1 signaling in NSCs after TBI. However, the ketamine treatment did not enhance the level of post-traumatic neurogenesis, but instead showed some potential in promoting functional recruitment of post-injury born neurons. Further studies are needed to illustrate the alternative mechanisms underlying the beneficial effects of ketamine on spatial learning function improvement post-trauma.

Materials and methods

Animal care

Male C57BL/6 mice (n=110) at the age of 8 weeks were purchased from The Jackson Laboratory and were housed and cared as described in chapter 2. All procedures were approved by Indiana University Animal Care and Use Committee.

Controlled cortical impact

Mice were subjected to sham or CCI injuries at the age of 8 weeks, and surgeries were performed as described in chapter 2. Injury parameters were set at 3.5 m/s, 1.0 mm, and 0.1 s.

Drug administration

Ketamine was given 3 times daily at 4 h apart (10mg/kg in sterile water i.p.) for 2 weeks after injury starting at 24 h after injury (n=30, Figure 4.1 A). Delayed paradigm was given with similar strategy except the first 2 doses were skipped, so the treatment started at 32 h after injury and also lasted for 2 weeks (n=20, Figure 4.2 A). BrdU (50mg/kg in sterile water, i.p.) was given once per day with the third ketamine injection for 2 weeks after injury (n=40).

Morris water maze test

Spatial learning and memory function was tested via Morris water maze test followed the protocol described by Vorhees and Williams (Vorhees and Williams, 2006). Briefly, a 103 cm diameter circular water tank with a 10 cm diameter circular platform was used in the experiments. The water was colored opaque by a non-toxic soluble white paint, and the platform was submerged 1 cm underwater. Water temperature was adjusted to 18 °C. Mice in the water tank was recorded, and their routes were traced and analyzed by a TopScan software (CleverSys Inc.). The test consisted of 5 days training and probe trial 1 day after last training. In each day of training, every mouse was subjected to 4 successive trials with pseudo random start positions listed in Table 3, and the inter-trial interval was 1 min. For each trial, the mouse was released at designated start position facing the water tank wall. When the mouse located the platform and stayed on the platform for 3 s successively within 60 s of the test, it was considered escaped. If the mouse did not locate the platform within 60 s, the test

Table 3: Start positions for Morris water maze test.

	Day1	**Day2**	**Day3**	**Day4**	**Day5**
Trial 1	N	SE	NW	E	N
Trial 2	E	N	SE	NW	SE
Trial 3	SE	NW	E	N	E
Trial 4	NW	E	N	SE	NW

Adapted from Table 1 (Vorhees and Williams, 2006).

was stopped, and the mouse was guided to the platform and left on the platform for 15 s. After each trial, the mouse was put in a cage provided with towels, placed on a heating pad, and left in the cage for 1 min before the next trial. After all 4 trials, the mouse was left in a warm cage until its fur was dry and was placed back to the home cage. On the sixth day, the platform was removed from the water tank, and each mouse was released at the same novel start position (NE). They were tested in the water maze for 60 s, with their routes tracked and analyzed. The average time to find the platform was calculated from 4 trials each day of each mouse. The escape time on day 1 to day 5 was plotted to represent spatial learning function. The time spent in each quadrant on day 6 was calculated, time spent in the target quadrant was compared to the other three quadrants to show spatial memory function. Data were shown as average ± standard error.

Immunohistochemistry and cell counting

Brain tissues were collected and processed to immunostaining as described in chapter 2. To quantify mTOR activity in NSCs after TBI, 3 epicentral sections were processed to immunostaining against Sox2 and pS6. Sox2 positive cells in the SGZ and Sox2 pS6 double positive cells in the SGZ were counted. The ratio of double positive cells in the Sox2 single positive cells was calculated. Activation of mTOR in NSCs were expressed as ration of mTOR active NSCs in total NSCs. To quantify post-traumatic neurogenesis, neuronal activity, and active post-injury born neurons, every sixth section covering the whole hippocampus were

subjected to immunostaining against BrdU, NeuN, and c-fos. The numbers of BrdU NeuN double positive cells, c-fos NeuN double positive cells, and BrdU c-fos NeuN triple positive cells were counted. Contours of GCL were drawn, and the area of GCL was measured. The cell density of post-injury born neurons were calculated by dividing the total number of BrdU NeuN double positive cells by the volume of GCL and expressed as cells/mm^3. The numbers of c-fos NeuN and BrdU c-fos NeuN positive cells were calculated by multiplying the counted number by 6 and shown as total cells per dentate gyrus.

Statistical analysis

Behavioral data were analyzed by SPSS via repeated measures ANOVA followed by *post hoc* test. Histological data were analyzed by SPSS via two-way ANOVA followed by *post hoc* test. Significance was set at $p<0.05$.

Results

Ketamine treatment improved spatial learning function at long-term time point

To assess if ketamine treatment can improve cognitive function recovery after TBI, I treated sham and TBI animals with a sub-anesthetic dose of ketamine (10mg/kg, i.p.) with a strategy of 3 doses every day, 4 h apart, for 14 days starting at 24 h after injury. I assessed the spatial learning and memory function of the mice 5 days and 33 days after the treatment (19 days and 47 days after injury) in Morris water maze (Figure 4.1 A).

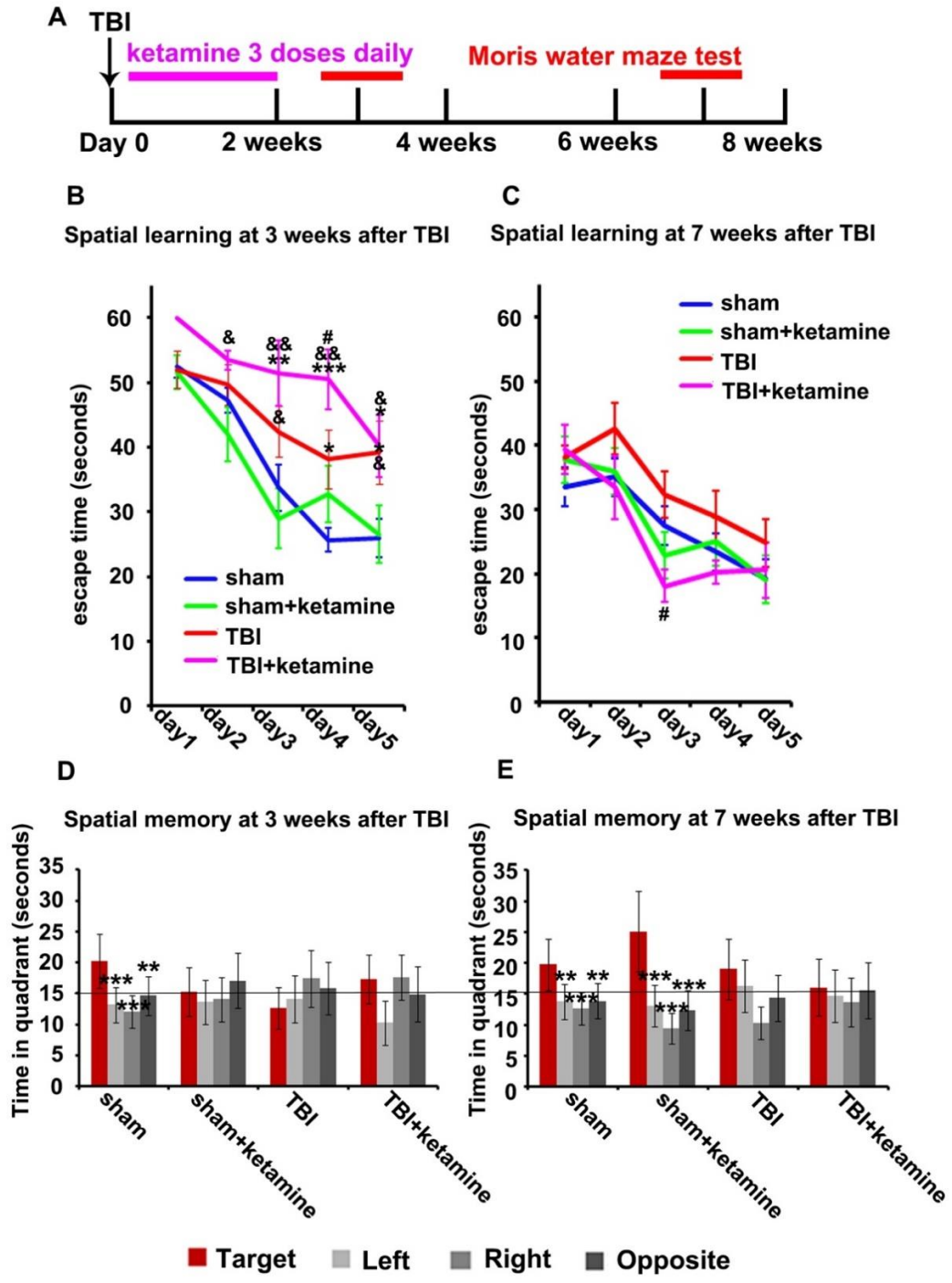


Figure 4.1: Ketamine treatment improves long-term spatial learning function recovery.

(A) A schematic shows the experimental design. Ketamine treatment starts at 24 h after injury. (B-C) Spatial learning function tested by a 5-day training paradigm

in Morris water maze test at 3 weeks (B, n=21 for sham, n=15 for sham+ketamine, n=14 for TBI, n=10 for TBI+ketamine) and 7 weeks (C, n=23 for sham, n=15 for sham+ketamine and TBI, n=12 for TBI+ketamine) after sham or TBI surgery with or without ketamine treatment (*p<0.05, **p<0.01, ***p<0.001 vs. sham. #p<0.05, ##p<0.01, ###p<0.001 vs. TBI. &p<0.05, &&p<0.01, &&&p<0.001 vs. sham+ketamine). (D-E) Spatial memory function in a probe test 24 h after last training session in Morris water maze test at 3 weeks (D) and 7 weeks (E) after sham or TBI surgery with or without ketamine treatment (*p<0.05, **p<0.01, ***p<0.001 vs. target quadrant).

In the 5-day session of spatial learning, all groups exhibited spatial learning ability, shown as mice spent less and less time to locate the hidden platform regardless of their groups (Figure 4.1 B, C). Whereas, the performances differed among the four groups. At 3 weeks after injury, sham animals spent 52.5 s ± 1.7 s learning to find the hidden platform on the first day, gradually progressed, and ended the training session within 26.0 s ± 3.0 s on the fifth day (Figure 4.1 B). Compared to sham animals, CCI injured animals showed spatial learning functional deficits, as they started the training at similar level on the first day (52.0 s ± 2.9 s, p=0.890 vs. sham), gradually progressed but in a slower manner, and spent more time learning to locate the hidden platform than sham animals, especially on day 4 (38.2 s ± 4.6 s in TBI vs. 25.7 s ± 1.8 s in sham, p=0.013) and day 5 (39.3 s ± 4.9 s in TBI vs. 26.0 s ± 3.0 s in sham, p=0.019) (Figure 4.1 B). At 7 weeks after injury, animals were tested in the MWM again, and they exhibited memory of the test by locating the platform faster at the first day of training session compared to the first day of the previous training session. Meanwhile, sham animals continued to progress in the test by spending 33.6 s ± 2.9 s finding the hidden platform on the first day and ending the training session within 19.2 s ± 2.2 s on the fifth day (Figure 4.1 C). Whereas spatial learning functional deficits in TBI animals persisted, thus they still performed worse than sham animals throughout the five-day session by starting slower on the first day (38.2 s ± 1.9 s) and ending slower on the fifth day (24.8 s ± 3.8 s), although the difference was not statistically significant. With ketamine treatment, sham animals performed consistently comparable to their non-treated counterparts

either in a short-term (Figure 4.1 B) or long-term (Figure 4.1 C) period after treatment. Whereas, ketamine treatment in TBI animals showed a transient adverse effect in a short-term period, as they performed even worse than TBI non-treated cohorts throughout the five-day training session, especially on day 4 (50.5 s $\pm$ 4.7 s in TBI + ketamine vs. 38.2 s $\pm$ 4.6 s in TBI, p=0.039, Figure 4.1 B). In a long-term period after treatment, however, ketamine treatment instead improves spatial learning function in injured animals, as treated animals performed consistently better than their non-treated TBI cohorts, significantly faster on day 3 (18.1 s $\pm$ 2.5 s in TBI + ketamine vs. 32.3 s $\pm$ 3.6 s in TBI, p=0.011, Figure 4.1 C).

In the 60 s probe test, sham animals showed spatial memory by spending much more time in the target quadrant, in which the hidden platform used to be, than in the other quadrants (Figure 4.1 D, E). Whereas, the TBI injured animals showed spatial memory functional deficits and failed to show preference for the target quadrant over the other quadrants in the probe test short-termly after injury (3 weeks, Figure 4. 1 D) or long-termly after injury (7 weeks, Figure 4.1 E). With ketamine treatment, sham animals exhibited a transient short-term spatial memory function defect, as they also failed to display preference for the target quadrant (Figure 4.1 D), which was spontaneously resolved in a long-term period (Figure 4.1 E). Whereas, for the TBI animals, ketamine treatment did not exhibit beneficial effects on spatial memory function, shown as the animals consistently spent comparable time in each quadrant and failed to show preference for target quadrant (Figure 4.1 D, E).

Collectively, TBI injured animals showed functional deficits in spatial learning and memory tests, and ketamine treatment improves spatial learning function recovery in a long-term manner while left spatial memory function unaffected.

Delayed ketamine treatment maintained long-term beneficial effect and eliminated short-term adverse effect

Although ketamine treatment showed a beneficial effect on spatial learning function recovery in a long-term manner, a transient adverse effect was observed shortly after treatment (Figure 4.1 B, D). I wondered if it is possible to sustain the beneficial long-term effects while minimizing the short-term adverse effects by modifying the treatment paradigm.

As previously mentioned in chapter 3, mTORC1 signaling is activated not only in NSCs after TBI. It is also activated in microglia and peaked at 24 h after injury, the time point I started ketamine treatment. The ketamine treatment might also exaggerate microglia reactivation, which may contribute to the adverse effect. To minimize the adverse effect, I applied a delayed treatment strategy as an alternative, in which I removed the initial 2 doses and started the treatment at around 32 h after injury (Figure 4.2 A). By the delayed treatment, ketamine treatment no longer exhibited adverse effect, but contrarily, treated animals showed better performances than non-treated TBI cohorts both short-termly and long-termly after injury (Figure 4.2 B, C), although statistical significance was only detected on day 1 (45.0 s $\pm$ 2.5 s in TBI + ketamine vs. 56.8 s $\pm$ 1.7 s in TBI,

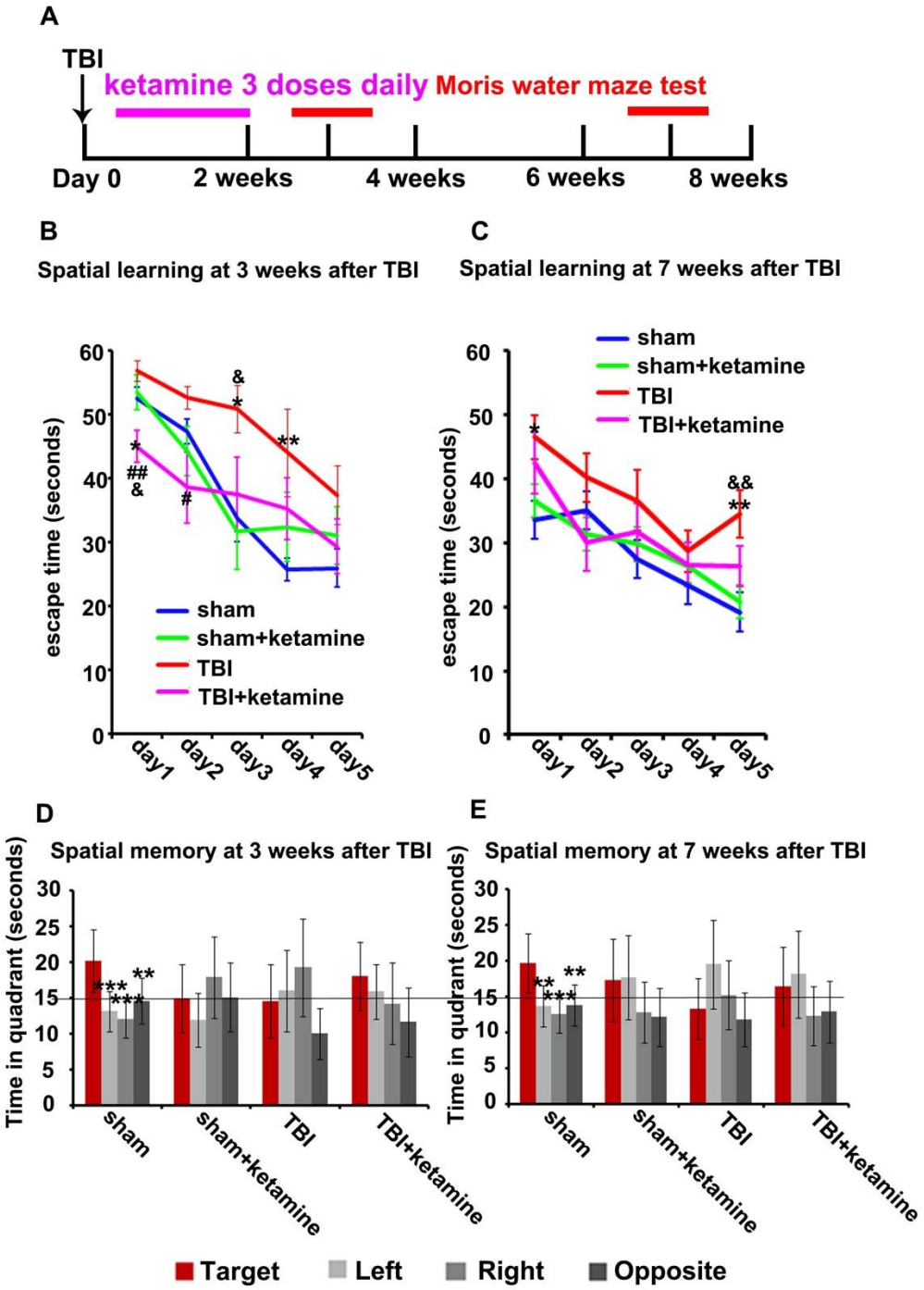


Figure 4.2: Delayed ketamine treatment alleviates short-term adverse effect on spatial learning function.

(A) A schematic shows the experimental design. Ketamine treatment starts at 32 h after injury. (B-C) Spatial learning function tested by a 5-day training paradigm

in Morris water maze test at 3 weeks (B, n=21 for sham, n=10 for sham+ketamine, n=8 for TBI, n=10 for TBI+ketamine) and 7 weeks (C, n=23 for sham, n=9 for sham+ketamine, n=10 for TBI, n=9 for TBI+ketamine) after sham or TBI surgery with or without delayed ketamine treatment (*p<0.05, **p<0.01, ***p<0.001 vs. sham. #p<0.05, ##p<0.01, ###p<0.001 vs. TBI. &p<0.05, &&p<0.01, &&&p<0.001 vs. sham+ketamine). (D-E) Spatial memory function in a probe test 24 h after last training session in Morris water maze test at 3 weeks (D) and 7 weeks (E) after sham or TBI surgery with or without delayed ketamine treatment (*p<0.05, **p<0.01, ***p<0.001 vs. target quadrant).

p=0.002, Figure 4.2 B), and day 2 (38.6 s ± 5.5 s in TBI + ketamine vs. 52.6 s ± 1.8 s in TBI, p=0.013, Figure 4.2 B) at 3 weeks after injury. In the probe test, delayed ketamine treatment was not able to improve spatial memory function recovery either in a short-term or long-term period as the previous paradigm, and injured animals still failed to display preference for target quadrant over other quadrants (Figure 4.2 D, E). Collectively, delayed ketamine treatment was able to remove the transient adverse effect on spatial learning function in injured animals while sustaining beneficial effects on spatial learning recovery after injury in a long-term period.

Ketamine treatment activated mTOR activity in NSCs after TBI

Since ketamine treatment was given systemically, it can target various mechanisms in the brain that may contribute to spatial learning recovery. I wondered if ketamine treatment benefits spatial learning function by activating mTORC1 signaling, and thus assessed mTORC1 activity in NSCs after TBI with ketamine treatment. I treated TBI injured animals with an intensive paradigm of ketamine treatment as a proof-of-concept, in which animals received ketamine injections (10mg/kg, i.p.) every 2 h for 24 h after injury (Figure 4.3 A).

By immunostaining against Sox2 and pS6, I quantified mTORC1 activation in NSCs after TBI. As previously described in chapter 3, mTORC1 activation in sham animals is low and mainly locates in the mature neurons (Figure 3.2 A and B, Figure 4.3 B). The activation of mTORC1 in NSCs is very limited, as only 2.6% ± 2.9% of Sox2 positive cells in the SGZ are also positive for pS6 (Figure 4.3 B,

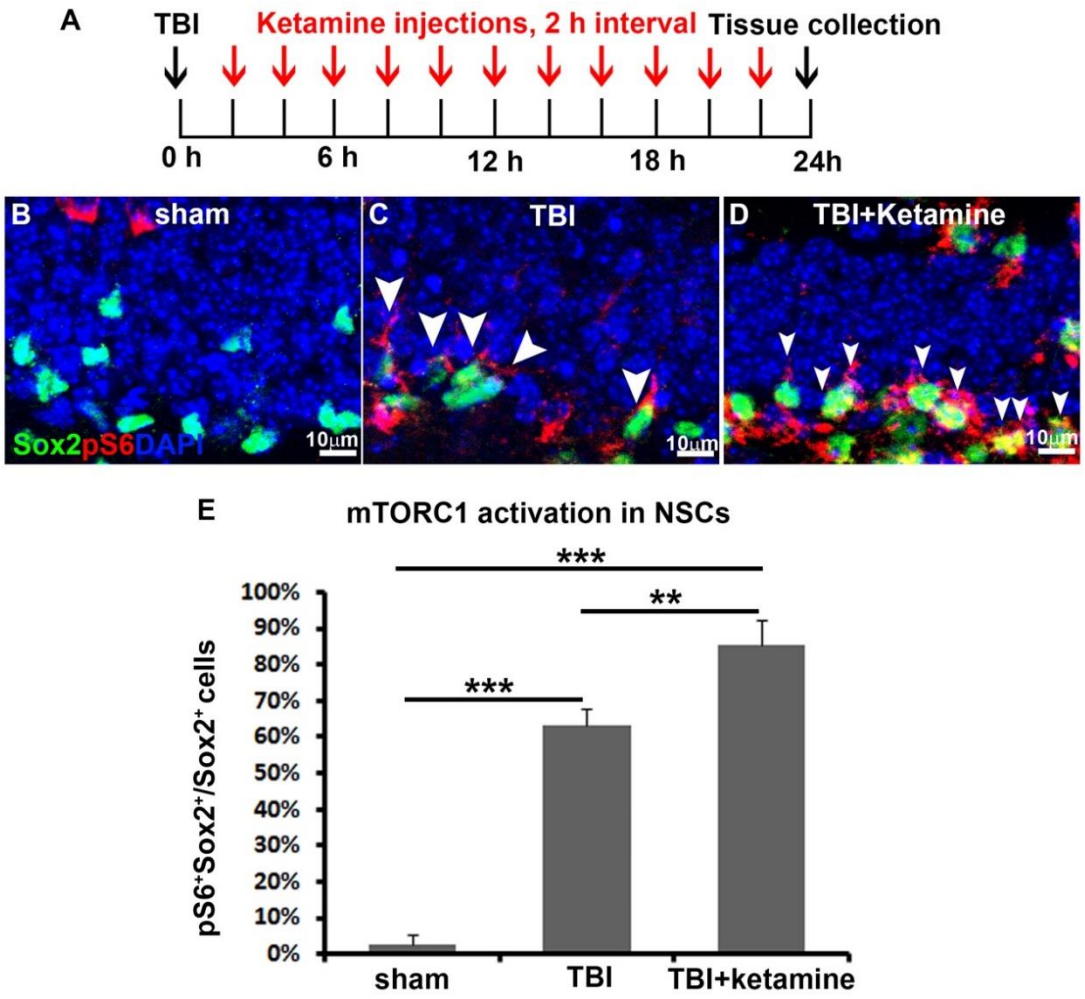


Figure 4.3: Ketamine treatment increases mTORC1 signaling activation in NSCs after TBI.

(A) A schematic shows the experimental design. (B-D) Immunostaining against pS6 (red) and Sox2 (green) shows mTORC1 activation in NSCs (indicated by white arrowheads) in sham (B), TBI (C), and TBI with ketamine treatment (D) animals. 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei shows GCL structure. (E) Percentage of pS6 and Sox2 double positive cells in total Sox2 positive cells in the SGZ (n=3 for sham and TBI+ketamine, n=4 for TBI, *p<0.05, **p<0.01, ***p<0.001).

E). At 24 h after TBI, I again observed dramatic increase of mTORC1 activity in the SGZ, and specific activation of mTORC1 in NSCs was significantly increased (indicated by white arrowheads, Figure 4.3 C), as 63.2% ± 4.7% of Sox2 positive cells in the SGZ were co-labeled with pS6 ($p<0.001$ vs. sham, Figure 4.3 E). Moreover, after ketamine treatment, mTORC1 activity was further promoted in the SGZ and specifically in NSCs (indicated by white arrowheads, Figure 4.3 D), leading to 85.2% ± 7.4% of mTORC1 positive cells in total Sox2 positive cells in the SGZ ($p=0.001$ vs. TBI, Figure 4.3 E). This data serves as a proof-of-concept that ketamine treatment can further promote mTORC1 activation in NSCs after TBI.

Ketamine treatment did not enhance post-traumatic neurogenesis nor alleviate post-injury born neuron mismigration

Since ketamine treatment was able to further promote mTORC1 activation in NSCs after TBI, it is potentially able to enhance post-traumatic NSC proliferation and subsequent neurogenesis, which may underlie the spatial memory function improvement long-termly after injury. To evaluate if ketamine treatment benefits spatial learning function by enhancing post-traumatic neurogenesis, I labeled cell proliferation with BrdU along with the delayed ketamine treatment and determined post-traumatic neurogenesis after Morris water test (Figure 4.4 A).

By immunostaining against BrdU and NeuN, post-traumatic neurogenesis was evaluated in each group (Figure 4.4 B-E). In sham animals, I again observed BrdU positive cells mainly in the GCL, and largely co-labeled with

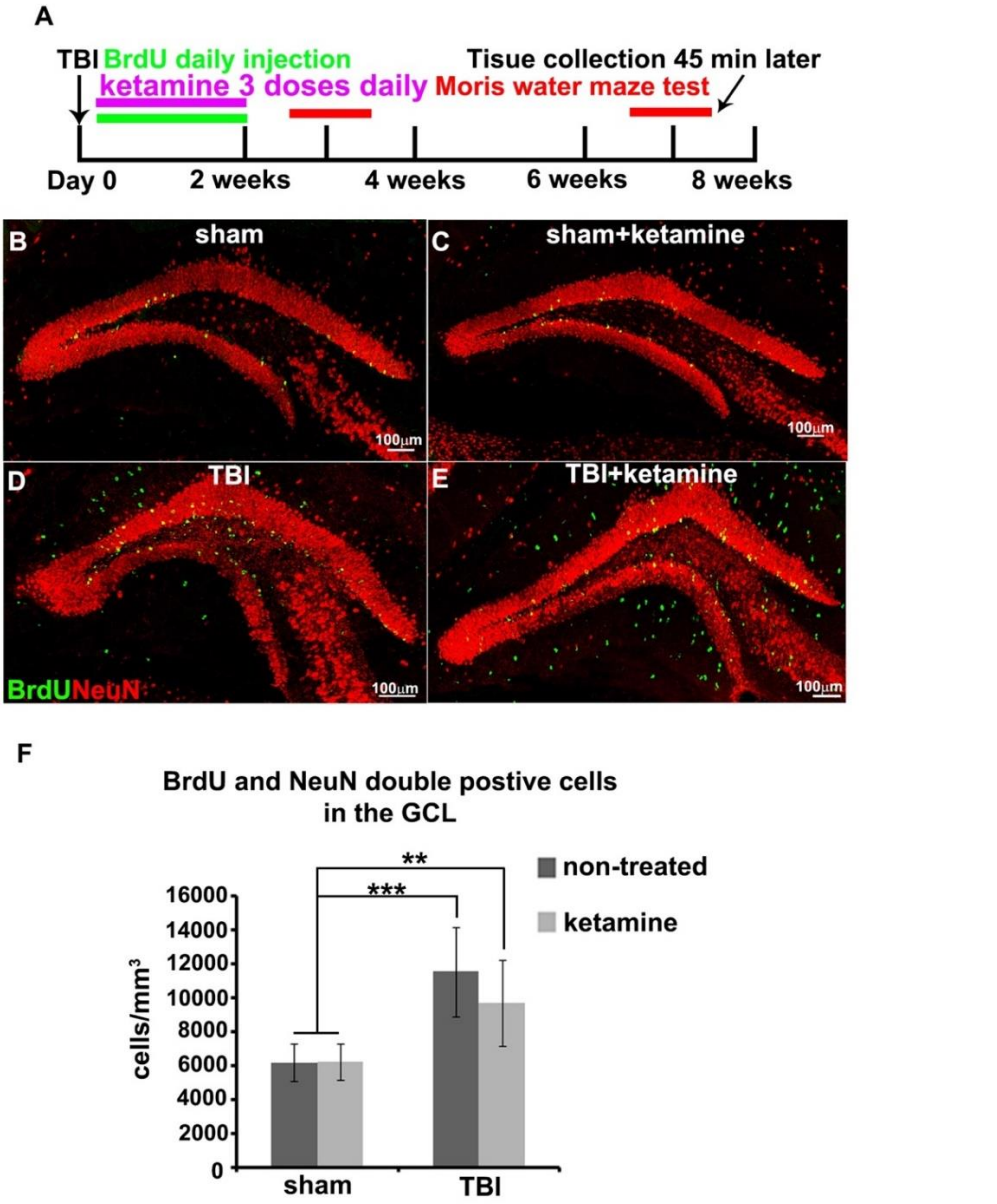


Figure 4.4: Ketamine treatment does not increase post-traumatic neurogenesis. (A) A schematic shows the experimental design. (B-E) Immunostaining against BrdU (green) and NeuN (red) shows post-traumatic neurogenesis in sham (B), sham with ketamine treatment (C), TBI (D), and TBI with ketamine treatment (E) animals. (F) Quantification of BrdU and NeuN double positive cells in the GCL (n=8 for sham and TBI, n=9 for sham+ketamine and TBI+ketamine, *p<0.05, **p<0.01, ***p<0.001).

NeuN (Figure 4.4 B). The baseline of neurogenesis was 6177 ± 1082 cell/mm^3 (Figure 4.4 F). At 7 weeks after TBI, I again observed a dramatic increase of BrdU positive cells throughout the hippocampal dentate gyrus, including a large proportion in the ML, which were not co-labeled with NeuN, corresponding to robust glial response (Figure 4.4 D). BrdU and NeuN double positive cells primarily located in the GCL, and neurogenesis was significantly elevated to 11532 ± 2662 cell/mm^3 by severe injuries ($p<0.001$ vs. sham, Figure 4.4 F). With ketamine treatment, I observed a similar pattern of BrdU labeling, mainly in the GCL in sham animals (Figure 4.4 C) and throughout the hippocampal dentate gyrus in TBI animals (Figure 4.4 E). In the GCL, with NeuN co-labeling, post-traumatic neurogenesis was assessed. Ketamine treatment did not change post-traumatic neurogenesis in either sham (6229 ± 1043 cell/mm^3 in sham + ketamine vs. 6177 ± 1082 cell/mm^3 in sham, $p=0.957$, Figure 4.4 F) or TBI animals (9709 ± 2532 cell/mm^3 in TBI + ketamine vs. 11532 ± 2662 cell/mm^3 in TBI, $p=0.068$, Figure 4.4 F).

Except for influences post-traumatic neurogenesis on the cell number level, ketamine might work through ameliorating abnormalities of post-injury born neurons to improve spatial learning function recovery. One of the issues reported on post-injury born neurons is their ectopic migration, as adult-born granule neurons are supposed to migrate from SGZ to inner GCL, but are observed to mismigrate to outer GCL and ML after injury (Ibrahim et al., 2016, Wang et al., 2016a). To assess if ketamine amends mismigration issue of post-injury born neurons, I further evaluated the localization of post-injury born neurons in the

GCL. In sham animals, majority of post-injury born neurons migrated from SGZ to inner GCL (indicated by white arrows, Figure 4.5 A), the baseline of post-injury born neurons located in the outer GCL is around 13.1% ± 2.6% (Figure 4.5 E). After injury, some post-injury born neurons migrated to and stopped in the inner GCL (indicated by white arrows), while a large number of them migrated to outer GCL (indicated by white arrowheads, Figure 4.5 C). The rate of misplaced post-injury born neurons in the outer GCL was dramatically increased to 32.1% ± 11.4% ($p<0.001$ vs. sham, Figure 4.5 E). However, ketamine treatment did not alter baseline of mismigration rate in sham animals (Figure 4.5 B, 12.6% ± 2.6% in sham + ketamine vs. 13.1% ± 2.6% in sham, $p=0.852$, Figure 4.5 E) or alleviate the mismigration issue in TBI animals (Figure 4.5 D, 27.8% ± 5.0% in TBI + ketamine vs. 32.1% ± 11.4% in TBI, $p=0.176$, Figure 4.5 E). Collectively, ketamine treatment did not increase post-traumatic neurogenesis or alleviate mismigration of post-injury born neurons.

Ketamine treatment potentially promotes activity of post-injury born neurons

To benefit functional recovery, not only an adequate number of neurons is needed, but also normal neuronal activity is required for any neurons to be recruited to the network. As an NMDAR antagonist, ketamine might benefit functional recovery by regulating neuronal activity in the hippocampus, so I evaluated neuronal activity both overall in the hippocampal dentate gyrus and specifically in post-injury born neurons after injury or sham surgery with or

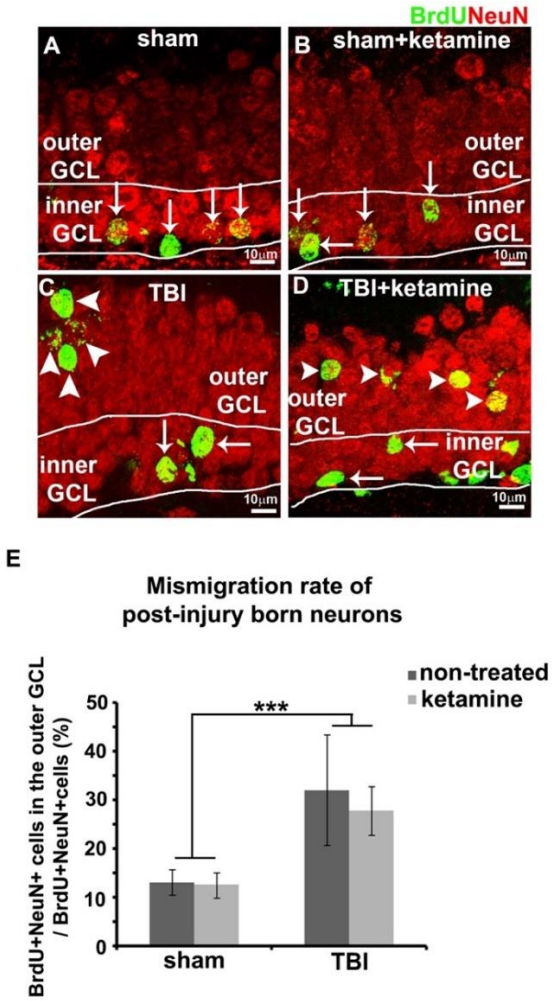


Figure 4.5: Ketamine treatment does not alleviate post-injury born neuron mismigration after TBI.

(A-D) Immunostaining against BrdU (green) and NeuN (red) shows post-injury born neurons with normal migration to the inner one third of GCL (indicated by white arrows) and with ectopic migration to the outer two thirds of GCL (indicated by white arrowheads) in sham (A), sham with ketamine treatment (B), TBI (C), and TBI with ketamine treatment (D) animals. (E) Percentage of mismigrated post-injury born neurons. (n=8 for sham and TBI, n=9 for sham+ketamine and TBI+ketamine, *p<0.05, **p<0.01, ***p<0.001)

without ketamine treatment. By immunostaining against c-fos and NeuN, I first evaluated overall neuronal activity in the hippocampal dentate gyrus 45 min after MWM probe test. In all groups, I observed that c-fos positive cells primarily located in the GCL, and greatly co-labeled with NeuN, indicating neuronal specific c-fos expression (Figure 4.6 A-D). This is consistent with the roles of c-fos as a functional marker for granule neurons in memory formation (Reijmers et al., 2007, Garner et al., 2012, Ramirez et al., 2013). By quantification, I found that the baseline of c-fos positive active neurons was 2940 ± 602 cells per dentate gyrus in sham animals (Figure 4.6 A, E). And the number was not altered either after injury (2799 ± 625 cells per dentate gyrus in TBI, p=0.778 vs. sham, Figure 4.6 E) or by ketamine treatment (3069 ± 759 cells per dentate gyrus in sham + ketamine, p=0.797 vs. sham, Figure 4.6 E). Not surprisingly, ketamine treatment did not change overall neuronal activity in the hippocampal dentate gyrus after injury either (3461 ± 766 cells per dentate gyrus in TBI + ketamine vs. 2799 ± 625 cells per dentate gyrus in TBI, p=0.201, Figure 4.6 E).

Since adult-born neurons undergo a process of morphological and functional maturation after birth, which can take up to months (Zhao et al., 2006), and ketamine treatment has been proved to promote immature neuron maturation at specific stage (Ma et al., 2017), I wondered if ketamine treatment possibly shapes newborn neuron maturation and thus enhances functional recruitment specifically of post-injury born neurons. By immunostaining against BrdU, c-fos, and NeuN, I then selectively assessed neuronal activity of post-injury born neurons. In sham animals, I detected a limited number of triple

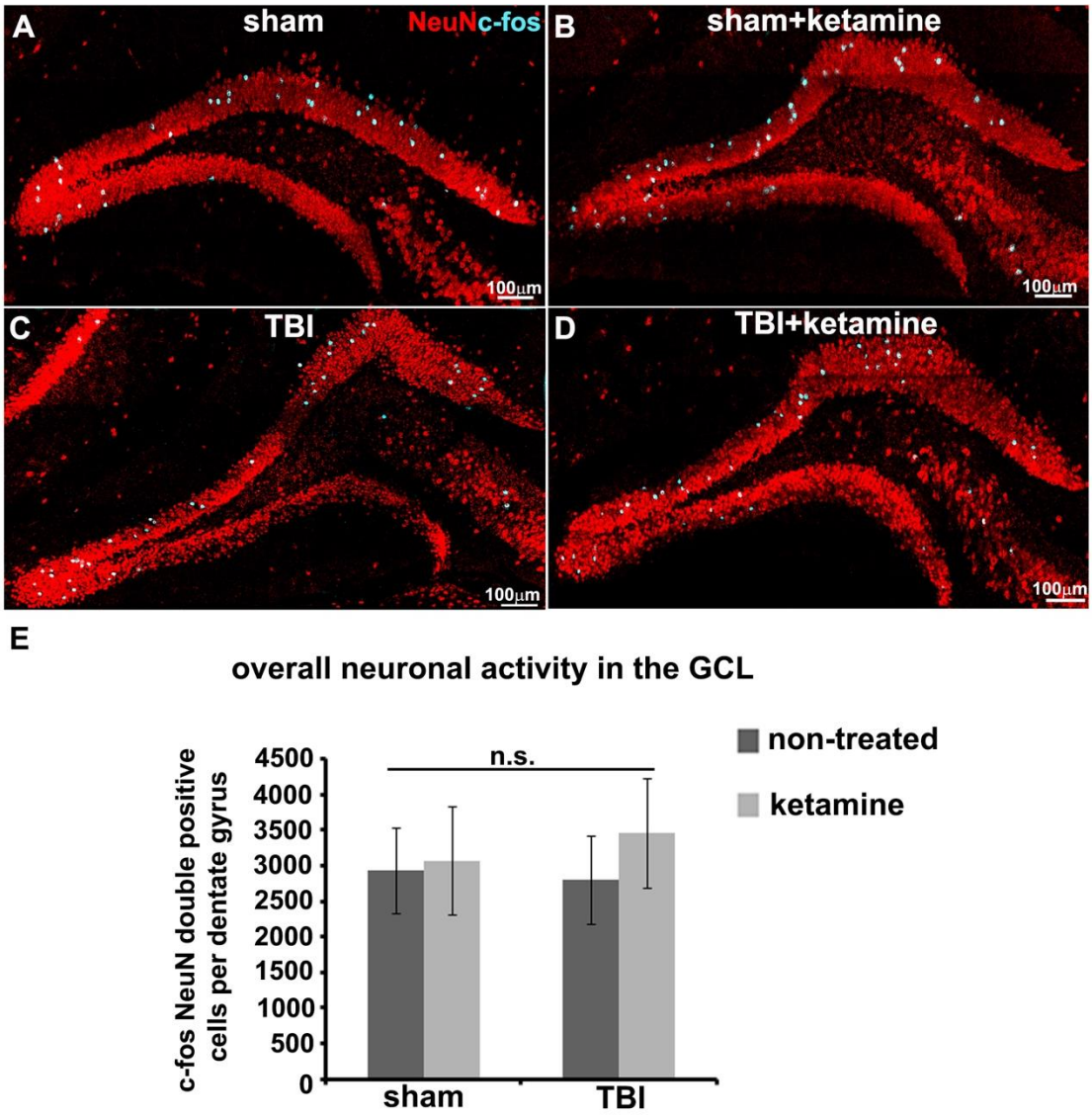


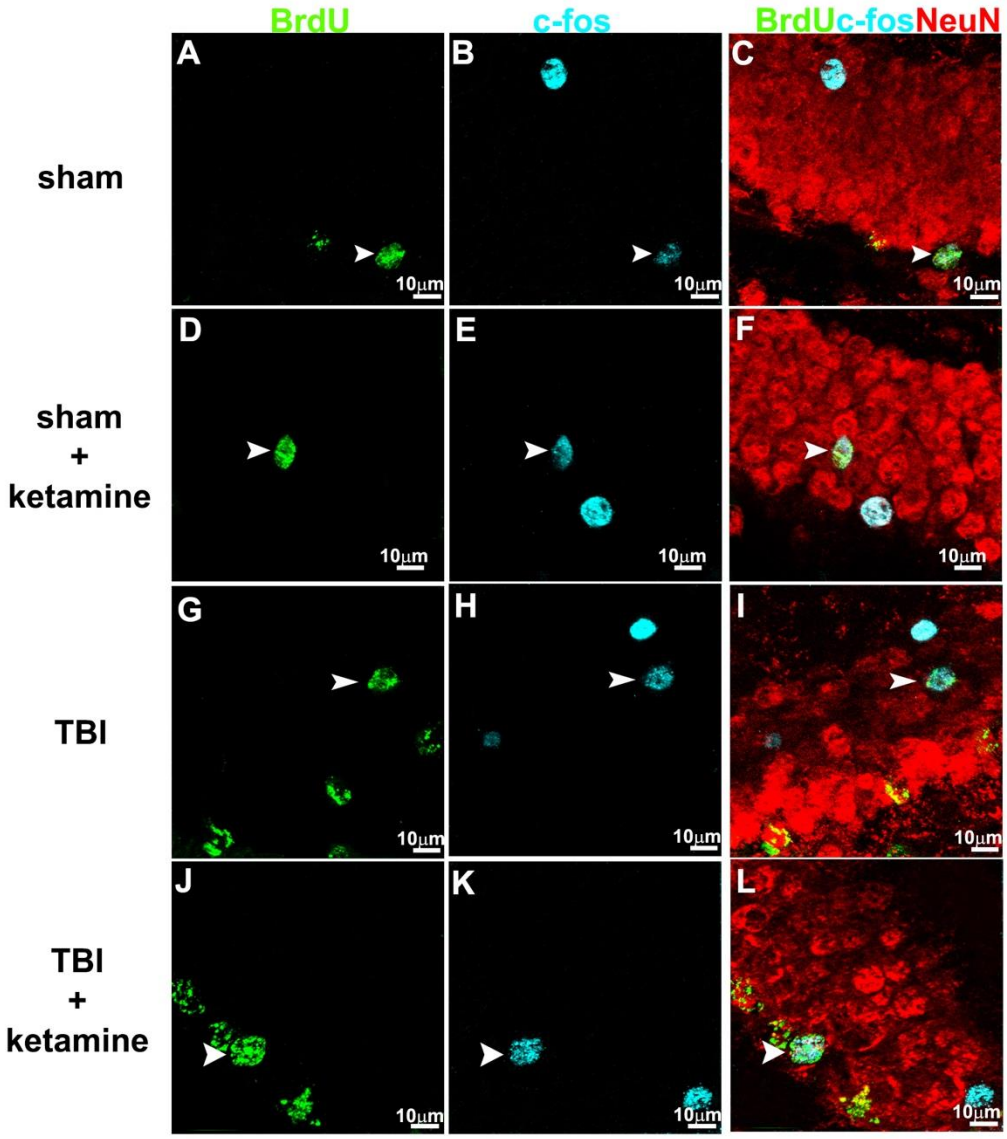
Figure 4.6: Ketamine treatment does not alter overall neuronal activity in the GCL.

(A-D) Immunostaining against c-fos (cyan) and NeuN (red) shows overall neuronal activity in the GCL in sham (A), sham with ketamine treatment (B), TBI (C), and TBI with ketamine treatment (D) animals. (E) Quantification of c-fos and NeuN double positive cells in the GCL (n=4 for each group, n.s.=no significance).

positive cells (indicated by white arrowheads, Figure 4.7 A-C, 14 ± 8 cells per dentate gyrus, Figure 4.7 M), consistent with functional integration of adult-born granule neurons in spatial network (Kee et al., 2007). With ketamine treatment, I detected a similar number of triple positive cells in sham animals (indicated by white arrowheads, Figure 4.7 D-F, 12 ± 6 cells per dentate gyrus in sham + ketamine, p=0.872 vs. sham, Figure 4.7 M). After injury, I found a slight but not significant increase of post-injury born neuron activation (indicated by white arrowheads, Figure 4.7 G-I, 18 ± 2 cells per dentate gyrus in TBI, p=0.631 vs. sham, Figure 4.7 M). With ketamine treatment, the number of triple positive cells was further increased and thus suggested a potential promotion in functional recruitment of post-injury born neurons (indicated by white arrowheads, Figure 4.7 J-L, 32 ± 10 cells per dentate gyrus in TBI + ketamine, p=0.165 vs. TBI, Figure 4.7 M). The numbers of triple positive cells in animals of all groups were very limited, which impeded the power of statistical analysis. Thus, further investigations on the activation of post-injury born neurons are needed to validate if injury itself with or without ketamine treatment indeed influences the functional recruitment of post-injury born neurons or not. Moreover, it would be interesting to study whether the functional recruitment of post-injury born neurons is regulated by hippocampal dependent behavior tasks or not.

Discussion

In order to take advantage of the molecular mechanism, namely mTORC1 mediated post-traumatic NSC proliferation, that I have demonstrated in chapter


BrdU
c-fos
BrdUc-fosNeuN
A
B
C
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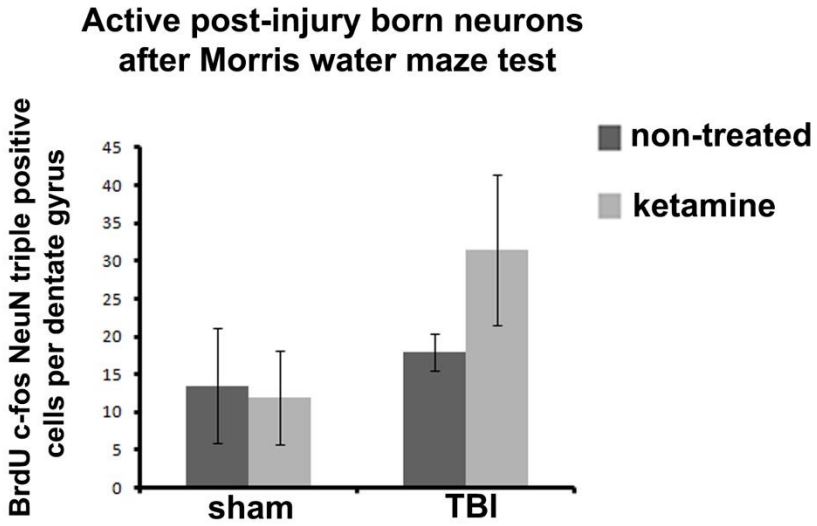
Active post-injury born neurons
after Morris water maze test
BrdU c-fos NeuN triple positive
cells per dentate gyrus
45
40
35
30
25
20
15
10
5
0
sham
TBI
non-treated
ketamine

Figure 4.7: Ketamine treatment shows a trend of promoting functional recruitment of post-injury born neurons.

(A-L) Immunostaining against BrdU (green), c-fos (cyan), and NeuN (red) shows active post-injury born neurons (indicated by white arrowheads) in sham (A-C), sham with ketamine treatment (D-F), TBI (G-I), and TBI with ketamine treatment (J-L) animals. (M) Quantification of BrdU, c-fos, and NeuN triple positive cells in the GCL (n=4 for each group).

3, sub-anesthetic anti-depressant dose of ketamine was used as an mTORC1 activator. Although ketamine has been used in the clinics since the 1970s for anesthesia and analgesia treatments as an NMDAR channel blocker, its role as a potent mTORC1 activator at sub-anesthetic dose was not discovered until the 2000s. Since its anti-depressant effect first reported in a small human study (Berman et al., 2000), more studies with larger patient group sizes confirmed the use of low-dose ketamine (0.5mg/kg) infusion as a rapid anti-depressant therapy to treatment-resistant depression patients either as single dose treatment (Zarate et al., 2006), or as repeated-dose (aan het Rot et al., 2010). Although the rapid onset of anti-depressant effects within hours is largely attributed to its direct regulatory roles in glutamatergic neurotransmission, the sustained influence up to a week after a single dose has suggested involvement of synaptic plasticity (Duman et al., 2019). Following mechanistic studies revealed that dose-dependent activation of mTORC1 signaling and activity-dependent BDNF release are required for ketamine induced synaptogenesis mediated rapid and sustained anti-depressant effect (Li et al., 2010, Liu et al., 2012).

In the current study, I took advantage of the mTORC1 activator role of ketamine and demonstrated that a sub-anesthetic dose of ketamine treatment is beneficial for post-traumatic spatial learning functional improvement in mice (Figure 4.1, 4.2). This beneficial effect was correlated to mTORC1 signal activation in NSCs after TBI as expected (Figure 4.3). However, it did not increase post-traumatic neurogenesis (Figure 4.4) or alleviate post-injury born neuron mismigration (Figure 4.5). Noteworthily, a trend was observed in which

there was an increase in functional recruitment of post-injury born neurons and a further increase by ketamine treatment (Figure 4.7), potentially contributing to the functional improvement.

Interestingly, in my assessment of spatial learning and memory functions, I observed a preferential improvement of spatial learning but not memory performance after ketamine treatment (Figure 4.1 and 4.2). In set-up of the MWM test, the spatial learning function in the 5-day training period is more likely representing memory acquisition or formation process, whereas the spatial memory function assessed by the probe test 24 h after the training period is closely symbolizing the memory retrieval process (Nakazawa et al., 2004). Classically, it has been considered that environmental stimuli activate neurons in the EC and hippocampus through the circuit of entorhinal cortex layer II (EC II) $\rightarrow$ DG $\rightarrow$ CA3 $\rightarrow$ CA1 $\rightarrow$ entorhinal cortex layer V (EC V) to initiate memory formation, and the reactivation of neurons in the same circuit facilitates memory retrieval (Nakazawa et al., 2004). Recent studies have increased current understandings on the circuit and demonstrated that circuits for memory formation and memory retrieval largely converge while slightly diverge. A place-learning dependent neuronal activity pattern along the EC II $\rightarrow$ DG projection was demonstrated essential for memory formation in visually guided navigation, and also required for memory retrieval (Qin et al., 2018), proving EC II and DG as essential and shared hubs for the memory circuits. Whereas, by cell-specific optogenetic inhibition, a study has pointed out that the CA1 $\rightarrow$ EC V projection is selectively indispensable for memory formation, whereas successful memory

retrieval requires an additional hippocampal sub-region, dorsal subiculum (dSub), and exclusively depends on CA1 → dSub → EC V pathway (Roy et al., 2017). In the model of TBI, the EC is remote from the injury site and my colleagues and I have not detected obvious cell death or tissue alteration in this region, whereas the hippocampus is largely damaged with dramatic mature and immature neuronal death in the DG, and sparse CA1 and CA3 pyramidal neuron death (Zhou et al., 2012, Gao et al., 2008, Wang et al., 2016a). I have not selectively assessed the damage of the subiculum in the TBI model. Collectively, damage of the shared nodes in memory functions including DG → CA3 → CA1 is possibly contributing to both aspects of the memory function deficits after TBI, whereas I cannot rule out the possibility that disruption in the subiculum also occurs and it possibly contributes to memory retrieval issues. These studies together bring about the possibility that in the TBI model, ketamine treatment might only alleviate damages in shared circuit components, like DG, CA3 and/or CA1, but neglect retrieval components, the subiculum. Thus beneficial effects were only observed in the spatial learning, the memory formation aspect, but not the memory retrieval aspect of the MMW test. Additionally, to fulfill the goal of escaping water maze task, the EC-hippocampus circuit is an essential core element in the full network, while more brain regions are required, like the superior colliculus, posterior cingulate cortex, and caudate nucleus (Garthe and Kempermann, 2013). It has been recently described that an analysis of the heterogeneous strategies used by mice instead of simple analysis on the escape time can help determine if a defect in the MWM task is hippocampus-dependent

or not (Garthe and Kempermann, 2013). Thus, further detailed analysis of my behavior data by categorizing searching strategies might help us understand if ketamine benefits spatial learning function likely in a hippocampal circuit dependent manner or not.

Although I observed increased activation of mTORC1 signaling in NSCs by ketamine treatment after TBI, post-traumatic neurogenesis was not correspondingly increased. This can result from unchanged NSC proliferation and/or newborn immature neuron survival issue. As discussed in chapter 3, mTORC1 is required for TBI-enhanced NSC proliferation. However, it works by driving RGL NSCs out of quiescence to alert state, but not directly initiate proliferation. The entrance of proliferation potentially depends on secondary signals. It is possible that ketamine promoted mTORC1 signal is able to keep RGL NSCs at the alert state for a prolonged period, but the lack of stimulatory signal and/or existence of inhibitory signal impedes the further enhancement on proliferation. Although NSC proliferation is the first step to initiate neurogenesis, the survival of immature neurons is another step affecting neurogenesis. More than 75% of newborn neurons would not survive in basal conditions (van Praag et al., 1999, Tashiro et al., 2006), while the survival rate of newborn neurons can be increased in an enriched environment (van Praag et al., 1999). Survival of newborn neurons depends on NMDA receptor mediated circuit integration, and it is locally controlled by astrocytes (Tashiro et al., 2006, Sultan et al., 2015). Additionally, microglia also play critical roles in newborn neuron integration and survival, depending on their modes of activation (Ekdahl, 2012). Although my

colleagues' and my previous studies and others did not observe significant change on survival rate of newborn neurons after TBI (Wang et al., 2016a, Chen et al., 2016), I cannot rule out the possibility that ketamine treatment may also decrease newborn neuron survival by altering astrocyte and/or microglia activities, which may contradict the potential enhancement on NSC proliferation and lead to the unchanged neurogenesis.

Noteworthily, the beneficial effect I observed in spatial learning tests is most prominent around 5 weeks after the ketamine treatment, indicating the involvement of a delayed mechanism. It roughly matches the time needed for proliferated NSCs to differentiate and become mature neurons (Bond et al., 2015). This supports the potential contribution of post-injury born neurons to functional recovery. Correspondingly, I observed a trend of increased recruitment of post-injury born neurons with ketamine treatment (Figure 4.7), consistent with others' observations (Soumier et al., 2016). Collectively, the data suggests that low dose ketamine might partially function to promote post-injury born neuron recruitment to enhance functional recovery.

As discussed in chapter 3, mTORC1 signal is not only activated in NSCs after TBI. Instead, spared mature neurons, microglia and astrocytes all have mTORC1 activation at different time points post-trauma. By the systemic introduction of ketamine, I am highly likely boosting mTORC1 activation in various cell types, and any of them may positively or negatively participate in cognitive function recovery. Moreover, as an anti-depressant treatment, a sub-anesthetic dose of ketamine has been reported to activate various mechanisms

besides mTORC1 signal (Duman et al., 2019). Among them, enhanced BDNF release and synaptogenesis are also applicable mechanisms in the case of post-traumatic functional improvement with ketamine treatment. Further investigations with specific silencing of post-injury born via optogenetic or chemical genetic tools would help determine if post-traumatic neurogenesis is required for spatial learning and memory function recovery after injury, and if the beneficial effects of ketamine on the recovery depends on post-traumatic neurogenesis or not.

CHAPTER 5

CONCLUSIONS

Summary

Traumatic brain injury patients suffer from cognitive dysfunctions, especially learning and memory impairments (Salmond and Sahakian, 2005). Hippocampal neuronal loss largely contributes to the problem (Wolf and Koch, 2016). The discovery of adult NSCs in the hippocampus provides great promise of utilizing the neuroregenerative approach to compensate cell loss. The work in current studies emphasizes research directed toward improving cognitive function recovery after TBI by taking advantage of endogenous adult hippocampal NSCs mediated neurogenesis to achieve neuronal replacement.

Although it is widely agreed that NSCs respond to TBI by increasing proliferation (Chirumamilla et al., 2002, Braun et al., 2002, Ramaswamy et al., 2005, Sun et al., 2005, Gao et al., 2009a), it is not consistently reported on whether TBI affects neurogenesis. The results in chapter 2 have depicted the baseline of post-traumatic NSC proliferation, immature neurons survival, and post-traumatic mature neuron generation after different severities of TBI. My results demonstrated that mild TBI does not change NSC proliferation, immature neuron number, or mature neuron generation. Moderate TBI increases NSC proliferation, but not immature neuron, or newborn mature neuron number. Severe TBI further increases NSC proliferation, increases immature neuron number, and promotes post-injury mature neuron generation. I speculated the

difference originated from the different cell death patterns induced by varying injury severities. This part of the work partially explains the pre-existing controversy in the field and sets the foundation for utilizing the process for neuronal replacement.

Despite morphological and electrophysiological studies on individual post-injury born neurons' properties (Villasana et al., 2015, Sun et al., 2007, Emery et al., 2005), their anatomical and functional integration has not been well documented. In chapter 2, I used a pseudorabies viruses based neurocircuitry tracing system to assess if the injury environment alters the anatomical integration pattern of post-injury born neurons. The results demonstrated that post-injury born neurons not only receive signals from the well-known projections but also make connections with neurons in brain regions that have not been previously discovered innervating dentate gyrus. Additionally, post-injury born neurons have been recruited to function on its neurocircuit with neuronal activity in a standard housing condition and during spatial learning and memory behavior task. This part of work demonstrates the capacity of post-injury born neurons in network integration and supporting behavioral performances.

One of the obstacles impeding the application of endogenous adult NSC mediated post-traumatic neurogenesis is the lack of a molecular target. In chapter 3, the current study demonstrated that mTORC1 is required for TBI-enhanced post-traumatic NSC proliferation, and proved that mTORC1 is primarily required for TBI-enhanced RGL NSC proliferation. MTORC1 has been proposed to prime RGL NSCs to a *de novo* alert state, in which RGL NSCs are reversibly

released from quiescence, prepared for proliferation, but maintaining the ability to return to quiescence. This part of work provides a molecular target for utilizing endogenous NSCs mediated neurogenesis to compensate cell loss, and deepens current understandings on RGL NSC dynamics.

Several clinical trials have been targeting post-traumatic neurogenesis to seek cognitive function improvement, yet none have succeeded. In chapter 4, a sub-anesthetic dose of ketamine treatment, an FDA-approved widely used drug, has been applied and proved beneficial for spatial learning function recovery. Furthermore, ketamine treatment has shown an effect on mTORC1 pathway activation in NSCs post-trauma without increasing post-traumatic neurogenesis. Ketamine treatment can also potentially increase the functional recruitment of post-injury born neurons. This part of work suggests the potential of ketamine as a treatment against post-traumatic cognitive impairments and reveals potential mechanisms through activating mTORC1 in NSCs and/or increasing functional recruitment of post-injury born neurons.

Overall, the body of this thesis work proves the feasibility of compensating for neuronal loss in the hippocampus by functional post-injury born neurons. This work provides a potential molecular target to enhance endogenous neuroregenerative machinery and reveals the therapeutic effect of an FDA-approved drug in cognitive function improvement post-trauma.

Future directions

Although post-traumatic neurogenesis has been correlated to functional recovery (Lu et al., 2005, Kleindienst et al., 2005, Lu et al., 2003), no evidence has directly proven the essential roles of post-injury born neurons in the functional recovery. It is of great interest to further assess functional outcomes with optogenetic and/or chemical genetic silence on post-injury born neurons to prove the cause-and-effect relationship between post-traumatic neurogenesis and functional improvement. Moreover, hippocampal neuronal loss is not the only mechanism underlying cognitive functional deficits, for axonal injury and dendritic and/or synaptic degeneration also contribute (Wolf and Koch, 2016). Thus, post-traumatic neurogenesis is not the sole approach for functional recovery, axonal sprouting and dendritic and/or synaptic regeneration mediated network reorganization may also participate in functional restoration. It would also be important to determine to what degree post-traumatic neurogenesis contributes to behavioral restoration.

Since I have demonstrated the essential role of mTORC1 signaling in the TBI-enhanced NSC proliferation, it would be critical to investigate the extracellular signals that selectively activate the mTORC1 pathway in NSCs. This might benefit NSC specific treatment while minimizing effects on other cell types that also have activated mTORC1 after injury, which would help to avoid potentially converse influences. Additionally, the proposed alert NSCs features a *de novo* node of NSC activity regulation. Further investigations on their cellular metabolism level, transcriptional and translational statuses, and epigenetic

profiling would help better distinguish alert NSCs from quiescent and active NSCs. Moreover, the fate decision mechanism at alert state controlling their return to quiescence or initiation of proliferation would be of great interest, since this could bring about new therapeutic targets on neuroregeneration, as well as on regenerative medicine in other organs.

Although I chose ketamine as a pharmacological approach to target mTORC1 signaling in NSCs, there are other mechanisms that are potentially undergoing parallel activations. For instance, the neuroprotection and synaptogenesis actions of ketamine might also contribute to the functional improvement (Duman et al., 2019, Wang et al., 2017). Further investigations on the mechanisms of ketamine improved cognitive function recovery might benefit the therapeutic applications of ketamine on TBI patients. Since ketamine successfully activates mTORC1 in NSCs after TBI, it is of great interest to delineate the reason why neurogenesis is not subsequently enhanced. It would be helpful to study if ketamine failed to increase post-traumatic NSC proliferation, or the enhanced NSC proliferation is counteracted by survival issue, thus leads to unchanged post-traumatic neurogenesis.

Besides ketamine, there are other reagents proved to be capable of activating mTORC1 pathway. Although they are not FDA-approved drugs yet, it is worth testing them in animal research to provide alternative avenues. For example, the combination of IGF-1 and osteopontin (Liu et al., 2017), the small molecule PTEN inhibitor bpv (Walker et al., 2019), and a new small molecule NV-5138 in clinical trial led by Navitor Pharmaceuticals (Sengupta et al., 2019,

Walker et al., 2019) are good candidates as mTORC1 activators. Since mTORC1 is implicated in neuroregeneration not only in regard to NSC proliferation but also in terms of axonal regeneration (Liu et al., 2010, Park et al., 2008), treatments aiming on mTORC1 can possibly hit multiple targets. Assessments on these different aspects would benefit the evaluation of treatment efficacy.

Additionally, NSC proliferation is only the initial step of neurogenesis, while there are other critical steps of neurogenesis that worth targeting, like the survival of newborn neurons. As mentioned earlier, more than 75% of newborn neurons die after birth (Tashiro et al., 2006, van Praag et al., 1999), makes this step another target with great potential. It has been proved that a small molecule mimicking BDNF is effective in promoting newborn neuron survival after TBI (Zhao et al., 2016). A combinational approach targeting NSC proliferation and newborn neuron survival would be expected to amplify the benefits of post-traumatic neurogenesis.

Clinical translation

My current study provides a potential target for utilizing endogenous hippocampal neurogenesis to seek cognitive functional improvement post-trauma; however, it is not close to clinical translation yet. TBIs in animals are studied in a precisely controlled manner, in which injuries are delivered by the exact same parameters at the same injury site to animals at the same age, with the same gender and genetic background who are raised in the same environment. However, human TBI cases are largely heterogeneous. Several animal models have recapitulated

some key differences in human TBIs, such as diffusive versus focal injury, closed-head versus penetrating injury, blast wave versus contusion injury, and single hit versus repetitive injuries, roughly represented the diversity of injury patterns in human (Hajiaghamemar et al., 2019). My result shows that even different severities of injuries generated by the same injury model can produce different effects on post-traumatic neurogenesis, it would not be surprising if different injury mechanisms differently influence the process.

In addition to injury mechanisms, patient's age may also complicate injury progression and recovery. In clinical observances, young children are more likely to suffer from neurological complications after TBI (Giza and Prins, 2006, Maxwell, 2012). While the elderly population shows relatively inferior functional outcomes under similar injury severities (Stocchetti et al., 2012). Although the difference may be attributed to increase of some age-dependent cellular mechanisms, such as apoptosis, oxidative stress, and inflammation, an extra contributor may be the age-dependent regenerative capacity decline (Fan et al., 2003, Timaru-Kast et al., 2012, Sun et al., 2013). As mentioned in chapter 1, age itself negatively regulates neurogenesis. Juvenile animals have a higher neurogenesis baseline compared to adults, while aged animals show a drastic decline in neurogenesis (Sun et al., 2005, Jinno, 2011). However, how age affects injury progression and post-traumatic neurogenesis has not been widely studied. Most current studies on post-traumatic neurogenesis were conducted in adult animals, and few have focused on pediatric or geriatric TBIs. TBI happens to immature brains, including in childhood and adolescence; this can cause

profound issues in neural network development that may result in long-lasting complicated neurological and cognitive dysfunctions. Thus it is essential to extend studies on post-traumatic neurogenesis to pediatric settings and exploit selective strategy to target development rescue. A pediatric TBI study in rodents compared post-traumatic SVZ neurogenesis when injury happened at different developmental stages, and reported that post-traumatic cell proliferation in the SVZ increased in immature brains, but the response sharply declines as age (Covey et al., 2010). Sun and colleagues have similarly detected a greater post-traumatic cell proliferation increase in the SGZ in juvenile rats compared to adults after a moderate FPI trauma (Sun et al., 2005). While the Nobel-Haeusslein group reported a decease in post-traumatic cell proliferation and neuronal differentiation at relatively same age in juvenile animals sub-acutely after injury (Potts et al., 2009). Collectively, current data suggests that immature brains respond to TBI robustly in the acute phase, but may suffer from sustained neurogenesis impairment chronically, potentially contributing to the profound neurological and cognitive dysfunctions after pediatric TBI. While for geriatric TBI, despite the remarkable prevalence of TBI in the elderly population, much less efforts were made to study how NSCs react to brain injuries in aged subjects. In other types of brain injuries, like stroke, even elderly patients exhibited increased NSC proliferation (Macas et al., 2006). TBI is highly likely able to stimulate NSC proliferation in elderly subjects, although the injury mechanisms vary. More endeavors should be made towards understanding how

NSC responses to TBI in the elderly group, and whether neuronal differentiation and survival happen.

In line with current attentions with gender differences, a few studies have also reported differences in post-traumatic neurogenesis in males versus female animals (Cahill et al., 2018, Brydges et al., 2018). Collectively, by precisely assessing the effects of age and gender on post-traumatic neurogenesis, it would be possible to develop more specified treatment strategies targeting individual situations to enhance neural repair and approach personalized medicine in the future.

More importantly, neuronal loss is not the only issue after TBI, and regeneration is not the only key to a cure to TBI. Although cognitive dysfunction is one of the most commonly complained symptoms post-trauma, other physical and neurobehavioral deficits are also widely reported and can persist chronically after injury. While these deficits are not discussed in the current study, they are also urgent problems in TBI research. There would not be a single silver bullet for TBI treatment, and combinational therapy is anticipated to provide the optimal strategy. For example, once a TBI patient get access to medical help, after or simultaneously with stabilization of intracranial pressure and hemorrhage, neuroprotective efforts might be considered as the first line of defense against neuronal loss and axonal and/or dendritic degeneration. Subsequently, neuroregenerative approaches might be considered as reinforcements to repair the inevitable damages and restore the functional network. Together potentially with neurotrophic and angiogenic endeavors, the injured tissue would be

replenished with newborn mature neurons that can properly function and adequately perfused by new neurovascular structure. Additionally, anti-inflammatory interventions should be expected accordingly. Extra approaches, like hypothermia, aimed at extending the therapeutic window for neuroprotection, will be beneficial for the remedy as well. Only if there is a comprehensive understanding on the temporal profile of events occurring after TBI and their specific roles in the pathophysiology of TBI, a guide can be developed to operate therapeutic interventions in an appropriate manner. It is a long journey to achieve the current understanding of TBI. It is urgently needed to carry on the studies and elucidate the detailed mechanisms behind pathophysiological events. Only by these means, will it be feasible o develop better strategies to precisely target the mechanisms, ameliorate the issues, and restore normal functions in the brain.

APPENDIX

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CURRICULUM VITAE

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Education

Ph.D. Anatomy and Cell Biology

2013-2019

Indiana University-Indiana University Purdue University-Indianapolis

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B.S. Biological Sciences

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Nankai University

Publications

Xiaoting Wang, Xiang Gao, Stephanie Michalski, Shu Zhao, Jinhui Chen, (2015) Traumatic brain injury severity affects neurogenesis in adult mouse hippocampus. ***Journal of Neurotrauma***, **33**(8): 721-733. doi:10.1089/neu.2015.4097

Shu Zhao, Alex Yu, **Xiaoting Wang**, Xiang Gao, Jinhui Chen, (2015) Post-injury treatment of 7,8-dihydroxyflavone promotes neurogenesis in the hippocampus of the adult mouse. ***Journal of Neurotrauma,*** **33**(22):2055-2064. doi:10.1089/neu.2015.4036

Sara Ibrahim, Weipeng Hu, **Xiaoting Wang**, Xiang Gao, Chunyan He, Jinhui Chen, (2016) Traumatic brain injury causes aberrant migration of adult-born neurons in the hippocampus. ***Scientific Reports**,* **6**, 21793. doi:10.1038/srep21793

Xiang Gao, **Xiaoting Wang**, Wenhui Xiong, Jinhui Chen, (2016) In vivo reprogramming reactive glia into iPSCs to produce new neurons in the cortex following traumatic brain injury. ***Scientific Reports**,* **6**, 22490. doi:10.1038/srep22490

Xiaoting Wang, Pich Seekaew, Xiang Gao, Jinhui Chen, (2016) Traumatic Brain Injury Stimulates Neural Stem Cell Proliferation via Mammalian Target of Rapamycin Signaling Pathway Activation. ***eNeuro,* 3**(5) e0162-16.2016: 1-14. doi:10.1523/ENEURO.0162-16.2016

Xiaoting Wang*, Jennifer Lynn Romine*, Xiang Gao, Jinhui Chen (2016) Aging impairs dendrite morphogenesis of newborn neurons and is rescued by 7, 8-dihydroxyflavone. ***Aging Cell***, **16**(2):304-311. doi:10.1111/acel.12553

Shu Zhao, **Xiaoting Wang**, Xiang Gao, Jinhui Chen (2018) Delayed and progressive damages to juvenile mice after moderate traumatic brain injury. ***Scientific Reports,* 8**:7339. doi: 10.1038/s41598-018-25475-9.

Sherleen Xue-Fu Adamson, Xubo Shen, Wendy Jiang, Vivien Lai, **Xiaoting Wang**, Jonathan Shannahan, Jason Cannon, Jinhui Chen, Wei Zheng (2018) Subchronic manganese exposure impairs neurogenesis in the adult rat hippocampus. ***Toxicological Sciences,*** Epub, doi: 10.1093/toxsci/kfy062

Book Chapter

Jinhui Chen, **Xiaoting Wang**, Xiang Gao. Neuroregenerative Medicine in TBI. In: Neurotrauma: A Comprehensive Textbook on Traumatic Brain Injury and Spinal Cord Injury, edited by Kevin Wang. Oxford University Press; 2018. p. 359 - 372.

Awards & Honors

2018. Best Poster Awards - 2018 International Neural Regeneration Symposium, Guangzhou, China

2016. Research Excellence Award – Annual Meeting of Indianapolis Chapter of Society for Neuroscience, Indianapolis, IN

2016. 2nd Prize - Sigma Xi Student Competition, Indiana University School of Medicine Chapter

2015. Travel Grant Award - National Neurotrauma Symposium, Santa Fe, NM

2015. Travel Grant Award – Indiana University School of Medicine

Research Experiences

Graduate Research

2014-2019

Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis, IN, 46202

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Research Assistant

2012-2013

State Key Laboratory of Experimental Hematology, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China

Mentor: Dr. Xiao Hu

Research Project: Extra-ribosomal functions of ribosomal proteins in regulating hematopoietic stem cells erythroid differentiation.

Undergraduate Thesis

2011-2012

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Mentor: Dr. Junjie Hu

Undergraduate thesis: Similarities and differences between GTPase-mediated ER fusion and mitochondrial fusion.

Presentations

Oral Presentations

“Mechanistic target of rapamycin signaling pathway activates quiescent neural stem cells and promote neuroregeneration after traumatic brain injury.” (July 2018) 2018 International Neural Regeneration Symposium, Guangzhou, China

“Response of neural stem cells to traumatic brain injury.” (May 2018) IU Spinal Cord and Brain Injury Research Group Spring Retreat, Martinsville, IN

“Childhood traumatic brain injury and the associations with behavioral problems in adolescence and adulthood.” (April 2018) IU Spinal Cord and Brain Injury Research Group Research Forum, Indianapolis, IN

“Does age matter? Age does impact post-traumatic brain repair.” (February 2018) IU Spinal Cord and Brain Injury Research Group Research Forum, Indianapolis, IN

“Cell cycle regulation in neuroregeneration following TBI.” (October 2017) IU Spinal Cord and Brain Injury Research Group Research Forum, Indianapolis, IN

“Remain quiescent or become active? A decision neural stem cells need to make after TBI.” (March 2017) IU Spinal Cord and Brain Injury Research Group Research Forum, Indianapolis, IN

“TBI calls quiescent neural stem cell on alert and entering cell cycle via activating mTOR signaling pathway.” (October 2016) 2nd Signature Center Initiative Retreat, Center for Spinal Cord and Brain Injury Research, Indianapolis, IN

“Traumatic brain injury stimulates neural stem cell proliferation via mammalian target of rapamycin signaling pathway activation.” (March 2016) Indianapolis Chapter of Society for Neuroscience, Indianapolis, IN

“Synaptic zinc deficiency affects neurogenesis in the hippocampus in aging mice.” (February 2016) IU Spinal Cord and Brain Injury Research Group Research Forum, Indianapolis, IN

“Molecular signaling mediated TBI-induced neural stem cell proliferation.” (October 2015) IU Spinal Cord and Brain Injury Research Group Research Forum, Indianapolis, IN

“Traumatic brain injury severity affects neurogenesis in adult mouse hippocampus.” (March 2015), IU Spinal Cord and Brain Injury Research Group Research Forum, Indianapolis, IN

Poster Presentations

Xiaoting Wang, Xiang Gao, Jinhui Chen “Mechanistic target of rapamycin signaling pathway mediates PDGFRα-induced reactive astrocytes proliferation after traumatic brain injury.” (May 2018) 24th Annual Kentucky Spinal Cord &

Head Injury Research Trust Symposium, Lexington, KY

Xiaoting Wang, Xiang Gao, Jinhui Chen “Mechanistic target of rapamycin signaling pathway mediates PDGFRα-induced reactive astrocytes proliferation after traumatic brain injury.” (April 2018) 2018 Indiana Traumatic Spinal Cord and Brain Injury Research Conference, Indianapolis, IN

Xiaoting Wang, Xiang Gao, Jinhui Chen “Mammalian target of rapamycin signaling pathway primes quiescent NSCs to promote neuroregeneration after traumatic brain injury.” (December 2017) Indiana University/Purdue University Joint Symposium on Spinal Cord and Brain Injury Research, Indianapolis, IN

Xiaoting Wang, Pich Seekaew, Xiang Gao, Jinhui Chen “Traumatic Brain Injury Stimulates Neural Stem Cell Proliferation via Mammalian Target of Rapamycin Signaling Pathway Activation.” (June 2016) Indiana Regenerative Medicine Symposium, Indianapolis, IN

Xiaoting Wang, Xiang Gao, Stephanie Michalski, Shu Zhao, Jinhui Chen “Traumatic brain injury severity affects neurogenesis in adult mouse hippocampus.” (July 2015) National Neurotrauma Symposium, Santa Fe, NM

Xiaoting Wang, Xiang Gao, Stephanie Michalski, Shu Zhao, Jinhui Chen “Traumatic brain injury severity affects neurogenesis in adult mouse

hippocampus." (May 2015) 21st Annual Kentucky Spinal Cord & Head Injury Research Trust Symposium, Louisville, KY

Xiaoting Wang, Pich Seekaew, Xiang Gao, Jinhui Chen, "mTOR signaling regulates neural stem cell proliferation in the adult hippocampus following traumatic brain injury." (May 2015) Indiana Regenerative Medicine Symposium, Indianapolis, IN